Haartman Institute
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TEMPERATURE-SENSITIVE SRC-TRANSFORMED MDCK CELLS IN 3D CULTURES AS A MODEL OF MALIGNANT TRANSFORMATION OF EPITHELIAL CELLS

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ACADEMIC DISSERTATION

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To my family

Abstract

The equilibrium between cell proliferation, differentiation, and apoptosis is crucial for maintaining homeostasis in epithelial tissues. In order for the epithelium to function properly, individual cells must gain normal structural and functional polarity. The junctional proteins have an important role both in binding the cells together and in taking part in cell signaling. Cadherins form adherens junctions. Cadherins initiate the polarization process by first recognizing and binding the neighboring cells together, and then guiding the formation of tight junctions. Tight junctions form a barrier in dividing the plasma membranes to apical and basolateral membrane domains.

In glandular tissues, single layered and polarized epithelium is folded into tubes or spheres, in which the basal side of the epithelial layer faces the outer basal membrane, and the apical side the lumen. In carcinogenesis, the differentiated architecture of an epithelial layer is disrupted. Filling of the luminal space is a hallmark of early epithelial tumors in tubular and glandular structures. In order for the transformed tumor cells to populate the lumen, enhanced proliferation as well as inhibition of apoptosis is required.

Most advances in cancer biology have been achieved by using two-dimensional (2D) cell culture models, in which the cells are cultured on flat surfaces as monolayers. However, the 2D cultures are limited in their capacity to recapitulate the structural and functional features of tubular structures and to represent cell growth and differentiation *in vivo*.

The development of three-dimensional (3D) cell culture methods enables the cells to grow and to be studied in a more natural environment. Despite the wide use of 2D cell culture models and the development of novel 3D culture methods, it is not clear how the change of the dimensionality of culture conditions alters the polarization and transformation process and the molecular mechanisms behind them.

Src is a well-known oncogene. It is found in focal and adherens junctions of cultured cells. Active src disrupts cell-cell junctions and interferes with cell-matrix binding. It promotes cell motility and survival. Src transformation in 2D disrupts adherens junctions and the fibroblastic phenotype of the cells. In 3D, the adherens junctions are weakened, and in glandular structures, the lumen is filled with nonpolarized vital cells. Madin-Darby canine kidney (MDCK) cells are an epithelial cell type commonly used as a model for cell polarization. Its-src-transformed variants are useful model systems for analyzing the changes in cell morphology, and they play a role in src-induced malignant transformation.

This study investigates src-transformed cells in 3D cell cultures as a model for malignant transformation. The following questions were posed. Firstly: What is the role of the composition and stiffness of the extracellular matrix (ECM) on the polarization and transformation of ts v-src MDCK cells in 3D cell cultures? Secondly: How do the culture conditions affect gene expression? What is the effect of v-src transformation in 2D and in 3D cell models? How does the shift from 2D to 3D affect cell polarity and gene expression? Thirdly: What is the role of survivin and its regulator phosphatase and tensin homolog protein (PTEN) in cell polarization and transformation, and in determining cell fate? How does their expression correlate with impaired mitochondrial function in transformed cells? In order to answer the above questions, novel methods of culturing and

monitoring cells had to be created: novel 3D methods of culturing epithelial cells were engineered, enabling real time monitoring of a polarization and transformation process, and functional testing of 3D cell cultures.

Novel 3D cell culture models and imaging techniques were created for the study. Attention was focused especially on confocal microscopy and live-cell imaging. Src-transformation disturbed the polarization of the epithelium by disrupting cell adhesion, and sensitized the cells to their environment. With active src, the morphology of the cell cluster depended on the composition and stiffness of the matrix. Gene expression studies revealed a broader impact of src transformation than mere continuous activity of src-kinase. In 2D cultures, src transformation altered the expression of immunological, actin cytoskeleton and extracellular matrix (ECM). In 3D, the genes regulating cell division, inhibition of apoptosis, cell metabolism, mitochondrial function, actin cytoskeleton and mechano-sensing proteins were altered. Surprisingly, changing the culture conditions from 2D to 3D affected also gene expression considerably. The microarray hit survivin, an inhibitor of apoptosis, played a crucial role in the survival and proliferation of src-transformed cells.

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Contents

List of original publications

This thesis is based on the following publications:

I Rahikkala Mira, Sormunen Raija and Eskelinen Sinikka (2001) Effects of src kinase and TGFbeta1 on the differentiation and morphogenesis of MDCK cells grown in three-dimensional collagen and Matrigel environments. J Pathol 195: 391-400.

II Töyli Mira, Rosberg-Kulha Linda, Capra Janne, Vuoristo Jussi and Eskelinen Sinikka (2010) Different responses in transformation of MDCK cells in 2D and 3D culture by v-Src as revealed by microarray techniques, RT-PCR and functional assays. Lab Invest 90:915-28.

III Töyli Mira, Rahikkala Maiju and Lehto Veli-Pekka. Failed lumen formation (‘filling of the lumen’) in 3D cultures of src-transformed MDCK cells; gene expression analysis of a cell culture model of early glandular carcinoma. Submitted.

The publications are referred to in the text by their roman numerals.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AJ	Adherens junction
ATP	Adenosine triphosphate
BSA	Bovine serum albumine
c-Src	Cellular Src
D	Desmosome
DAPI	4', 6-diamidino-2-phenylindole
D-MEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-Mesenchymal -Transition
EPP	Epithelial Polarity Program
FA	Focal adhesion
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein 5-isothiocyanate
HGF	Hepatocyte growth factor
IAP	Inhibition of apoptosis
IGF	Insulin-like growth factor
kD	kiloDalton
mAb	Monoclonal antibody
MDCK	Madin-Darby Canine Kidney
MOMP	Permeabilization of the mitochondrial outer membrane
p120ctn	p120-catenin
pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI3-kinase	Phosphatidylinositol 3-kinase, phosphoinositide 3-kinase
PKB	Protein kinase B
PP2	4-amino-5-(4-chloro-phenyl)-7-(t-butyl) pyrazolo [3, 4,-5] pyrimidine
PMSF	Phenylmethylsulfonyl fluoride
PtdIns (3, 4, 5) P3	Phosphatidylinositol (3, 4, 5)-trisphosphate, PIP_3
PtdIns (4, 5) P2	Phosphatidylinositol (4, 5)-bisphosphate, PIP_2
PTEN	Phosphatase and tensin homolog protein
ROI	Region of interest
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SH3	Src homology 3
TCA	tricarboxylic acid
TGFβ	Transforming growth factor β
TRITC	Tetramethylrhodamine isothiocyanate
ts-Src	Temperature-sensitive Src
v-Src	Viral Src
VEGF	Endothelial growth factor

1 Introduction

Epithelial cells delineate skin and organs and can form sheet-like, tubular or glandular structures in different organs. Cell polarity is crucial for the functioning of epithelial cells. The formation of tubular structures follows a complicated program of epithelial cell morphogenesis, i.e. the epithelial polarity program (EPP) (Tanos and Rodriques-Boulan, 2008).

Most advances in cell and cancer biology have been achieved in *in vitro* studies, where normal and malignant cells are grown in two-dimensional (2D) cultures. In 2D, the cells are grown as a mat or a monolayer; the basal side is attached to the cell culture flask or glass, and the apical side faces the cell culture medium. However, the 2D culture conditions are not able to recapitulate the differentiation programs typical of epithelial structures *in vivo*. The 2D systems have serious limitations when used to study cellular differentiation as part of organ structure and functions (Seeberger et al., 2009).

Several three-dimensional (3D) cell culture systems have been developed in order to analyze the key processes of tube morphogenesis. The Madin-Darby canine kidney (MDCK) epithelial cell system is an excellent model for investigating epithelial morphogenesis *in vitro*. MDCK cells embedded in extracellular matrix (ECM) gel form cysts, i.e. spherical epithelial monolayers enclosing a central fluid-filled lumen (Nelson and Bissell, 2006). Results from such *in vitro* models have led to a general model for tube morphogenesis. Currently, only cell cultures in which the cells are grown in 3D matrices are able to form spherical or glandular structures similar to those *in vivo* (Debnath and Brugge, 2005; Pampaloni et al., 2007). They should thus be considered as legitimate *in vitro* representations of polarization and differentiation *in vivo*.

Carcinomas derive from epithelial cells and form by far the most important group of human cancers. Loss of normal architecture and cell polarization is typical to them (Tanos and Rodriques-Boulan, 2008). It is obvious that studying carcinoma formation *in vitro* would benefit from 3D systems which are able to closely mimic the conditions and processes seen *in vivo* (Debnath and Brugge, 2005; Pampaloni et al., 2007).

Tumorigenesis is a multistep process starting from a premalignant state and proceeding stepwise to malignancy. In order to develop from normal to malignancy, a cell must be able to proliferate, evade apoptosis, and become more motile. In epithelial tissues having tubular of glandular structures, loss of cellular polarity and filling of the luminal space with transformed cells is a hallmark of early epithelial tumors. This phenomenon is seen in hyperplastic glandular tissues in which the lumen is filled with vital cells (Martin-Belmonte and Mostov, 2008; Hanahan and Weinberg, 2000).

E-cadherin is the central component of the cell-cell adhesions that keep the neighboring epithelial cells together. It also determines the formation of the basolateral walls of a polarized cell. Next to cell adhesion, it also takes part actively in cellular signaling. Cadherins are considered to be tumor suppressors. In fact, the expression of cadherins is downregulated in many cancers (Yamada et al., 2005).

Integrins, a family of dimeric transmembrane proteins, are present in specialized plasma membrane domains that mediate attachment of cells to extracellular matrix. They are also physically linked to actin cytoskeleton. Due to these interactions, integrins

strongly regulate the orientation of the apicobasal axis and, consequently, the polarity of the cells.

Src-protein is a nonreceptor tyrosine kinase located both at adherens junctions at the basolateral walls and in focal adhesions at the basal surface of the cells. Thus, it can be anticipated that upon activation of src, as is the case in many cancers, enhanced activity of src could affect basolateral differentiation (cell-cell adhesions) and apico-basal axis by interfering with integrin-mediated interactions. Indeed, activation of src is associated with weakened cell-cell adhesion due to disintegration of the adherens junctions in cell culture models. Moreover, in src-transformed cells cultured in 2D, the cuboidal epithelial cell morphology becomes more flattened and fibroblastic in shape (Behrens et al., 1993).

One of the cell models used to study src-mediated transformation is the designated MDCK cell line that is transfected with a pp60src-gene, and encodes a src-protein that is temperature-sensitive. This ts v-src MDCK cell line was designed and established by Behrens et al. (1993). The cells show a strictly polarized epithelial phenotype at a non-permissive temperature of 40.5 ^{o}C at which the encoded src-protein is inactive. At permissive temperature (35 ^{o}C), the src protein is active and the cells acquire a fibroblast-like morphology displaying loss of cell-cell contacts and loss of vertical polarity. The change is transient and can be reversed by changing the temperature (Behrens et al., 1993). This provides an ideal model for analyzing the early steps in epithelial cell transformation induced by src.

Numerous studies have shown that culture dimensionality, whether 2D or 3D, and the quality and quantity of the matrix composition in 3D greatly affect the differentiation and polarization of epithelial cells in culture.

The aim of this study was to investigate the v-src transformation process and the mechanisms behind the loss of polarity and filling of the lumen observed in 3D cultures using ts v-src MDCK cells as a model for adenocarcinoma. Novel 3D culture and microscopy techniques were designed to meet the need to preserve and image the original 3-dimensional structures. In order to monitor the polarization and transformation process in real time, E-cadherin-GFP-ts v-src MDCK cell lines were established. Gene arrays were used to analyze changes in the expression levels of genes that are affected by changes in the dimensionality of the cell culture and by transformation by src.

2 Review of the literature

2. 1 Phenotypic features and mechanisms of cancer

Enormous advances have been made in cancer research during the past 40 to 50 years. Regardless of these advances, the multistep transformation process resulting in abnormal cell growth is still not completely understood. Cancer has been thought to originate from genetic alterations, such as mutations, deletions, rearrangements, as well as gene amplifications in the cancer cell genome that lead to abnormal expression of tumor suppressor genes and oncogenes. The genomic approach is well established and documented, but also other competing theories on the origins of cancer attempt to explicate the great variety of physiological changes observed in cancer tissues (Sonnenschein and Soto, 2008; Baker and Kramer, 2007; Soto and Sonnenschein, 2004; Seyfried and Shelton, 2010; Hanahan and Weinberg, 2000; 2011).

Malignant transformation can be initiated by numerous factors, e.g. radiation, chemicals, viruses or inflammation. Basically, any prolonged exposure to a provocative agent can potentially cause cancer (Loeb, 2001; Szent-Gyorgyi, 1977). "The oncogenic paradox" states that a very specific process, like cancer, can be initiated in very unspecific ways (Loeb, 2001; Szent-Gyorgyi, 1977; Sonnenschein and Soto, 2008; Seyfried and Shelton, 2010).

Starting from the clinical description of various cancers in the course of past centuries, cancer research in the early 20th century advanced to experimental and to *in vitro* studies. Step by step, they led to the discovery that all cancers have cellular phenotypic features that are common to all or most of them. A recent culmination of the efforts to find common defining features in all cancers is the schema of Hanahan and Weinberg (2000, 2011). They suggest that six essential alterations in cell physiology underlie malignant cell growth (Hanahan and Weinberg, 2000). The alterations were described as "the hallmarks of cancer" and include: 1) self-sufficiency in growth signals, 2) insensitivity to growth inhibitory (antigrowth) signals, 3) evasion of programmed cell death (apoptosis), 4) limitless replicative potential, 5) sustained vascularity (angiogenesis), and 6) tissue invasion and metastasis. Later on, the rearrangement of cellular energy levels and avoidance of immune response have been added to these hallmarks (Hanahan and Weinberg, 2011).

2.1.1 Phenotypic hallmarks of cancer

Self-sufficiency in growth signals

Normal cells need growth signals for proliferation. These signals are transmitted to the nucleus via cell signaling cascades after binding of a growth factor to a cognizant receptor.

Diffusible growth factors, extracellular matrix components, and cell-cell adhesion/interaction molecules can promote growth. Activation of proto-oncogenes to oncogenes can accrue cell independence from growth signals by mimicking, short-circuiting, or overriding normal growth signaling. This can be achieved by: 1) aberrant expression of growth factors, 2) activation of intracellular signaling pathways downstream of the ligand/receptor pair, 3) or enhanced intracellular response to growth signals (Hanahan and Weinberg, 2000).

Insensitivity to antigrowth signals

Multiple antiproliferative signals are involved in maintaining cellular and tissue homeostasis. The signals include both soluble and insoluble growth inhibitors. Physical contact between neighboring cells by interacting with or locking proteins inhibits cell division. Growth-inhibitory signals are received by transmembrane cell surface receptors coupled to intracellular signaling circuits.

Antigrowth signals inhibit cell division by removing the cells from the cell cycle or by arresting it. The cells can circumvent their ability to proliferate by tissue speciation and differentiation (Hanahan and Weinberg, 2000).

Insensitivity to growth signals can be caused by mutations or functional loss of tumor suppressor genes leading to anchorage independency (Cremona and Lloyd, 2009; Hanahan and Weinberg, 2011).

Evading apoptosis

In order for a tumor to grow in size, the cells must not only proliferate, but also evade programmed cell death called apoptosis. Mitochondria regulate the apoptotic signals by releasing cytochrome C to the cytoplasm. Members of the Bcl-2 protein family can function as either apoptotic (Bax, Bak, Bid and Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W). They act by modifying Cytochrome C release (Green and Reed, 1998).

One of the most important regulators of apoptosis is p53-protein, which is lost in many tumors. It is a tumor suppressor protein that can elicit apoptosis by upregulating the expression of proapoptotic Bax in response to sensing DNA damage. Bax in turn stimulates mitochondria to release cytochrome C. The final effectors of apoptosis include an array of intracellular proteases called caspases.

The PI3 kinase-AKT/PKB pathway is one of the major pathways which, when activated, counteracts apoptosis by inhibiting the action of pro-apoptotic BAD.

Cells can evade apoptosis in several ways. In many cancers, the function of tumor suppressor p53 is lost. On the other hand, the PI3-kinase pathway is hyperactive, leading to enhanced proliferation. Another mechanism by which cancer cells avoid apoptosis is inactivation or loss of Phosphatase and tensin homolog protein (PTEN), a phosphatase whose normal function is to negatively regulate the AKT/PKB signaling pathway (Thornberry and Lazebnik, 1998).

Limitless replicative potential

Cell growth is regulated not only by the extrinsic factors described above. Cells also have intrinsic machinery that restricts their life span after a predetermined number of cell cycles. This function is based on telomeres. In cancer cells, this restraint is overcome by the continuous expression of telomerase, which underlies the capacity of cancer cells to maintain telomeres.

Various normal human cell types are capable of 60–70 doublings. At every cell division, the telomeres are shortened. Telomere maintenance above the critical threshold is evident in virtually all types of malignant cells, enabling the cells to replicate without limits (Shay and Bacchetti, 1997).

Sustained angiogenesis

Sufficient provision of oxygen and nutrients is crucial for normal cell functioning and survival. The formation of new blood vessels is thus required for tumors to grow in size. In neoplasias, angiogenic capacity and ability is a prerequisite for maintaining growth (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997). Tumors secrete, for instance, endothelial growth factor (VEGF) and acidic and basic fibroblast growth factors (FGF1/2) to promote neovascularization (Veikkola and Alitalo, 1999).

Tissue invasion and metastasis

In most human cancers, cells migrate from the primary tumor, invade adjacent tissues and travel to remote sites to form new colonies. Remote colonies derived from the primary tumor are called metastases. Metastases cause 90% of human cancer deaths (Sporn, 1996). Invasion and metastasis are exceedingly complex processes which, in the case of epithelial cancers, such as carcinomas, are dependent on a phenomenon known as epithelial-mesenchymal-transition (EMT) (Aplin et al., 1998; Giancotti and Ruoslahti, 1999).

2.1.2 Molecular mechanisms of cancer

Oncogenes

The first oncogenes were detected in the 1970s in transforming retroviruses, and later in human tumors (Croce, 2008). Subsequent studies have shown that oncogenes are altered forms of normal genes which in normal cells encode proteins that are involved in controlling cell proliferation, apoptosis, and survival. Proto-oncogenes, as the normal variants of oncogenes are called, can be activated to become oncogenes by point mutation,

gene amplification or chromosomal rearrangements (Konopka et al., 1985; Tsujimoto et al., 1985; Croce, 2008).

The products of oncogenes can be roughly classified into six major groups according to the functions of their respective proto-oncogenes: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers, and apoptosis regulators (Croce, 2008). For instance, src is a non-receptor tyrosine kinase and a signal transducer which in normal cells has an important role in cellular physiology (Salgia and Skarin, 1998).

Figure 1 shows the basic principles of signal transduction, i.e., how an extrinsic factor can alter cellular behavior (Cummings, 2007).

In cancer, a proto-oncogene can be activated in such a manner that the cell acquires a competitive edge by promoting growth and/or survival and, consequently, expansion of the cell clone (Bishop, 1991). Among the activation mechanisms, translocations and mutations are often linked to the initiating events of tumorigenesis or to tumor progression. Gene amplification usually occurs later during tumor progression (Finger et al., 1986).

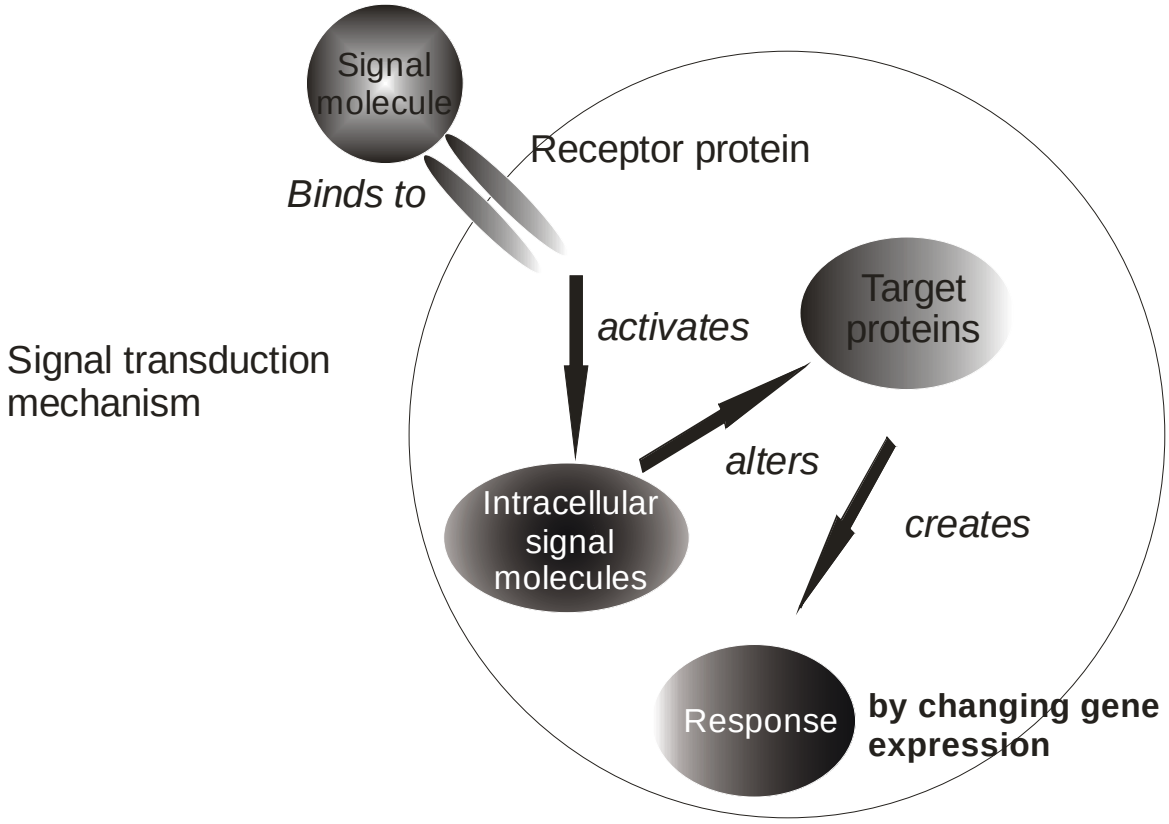


Figure 1 *General principles of signal transduction. A signal molecule binds to its specific receptor, leading to activation of an intracellular signaling cascade. The signaling cascade alters the target molecule and creates a response. (Modified from Cummings, 2007).*

Tumor suppressors

Tumor suppressor genes are normal genes that slow down cell division, repair DNA mistakes, and induce apoptosis after DNA damage or cell stress. Loss of or defective function of tumor suppressor genes leads to uncontrolled cell division or enhanced survival and, in this way, can lead to cancer. Many different tumor suppressor genes have been identified, including p53, BRCA1, BRCA2, APC, and RB1, PTEN, survivin and E-cadherin. P53 is mutated in almost half of all cancers (Seton-Rogers, 2009).

2.1.3 Cell death: Apoptosis, anoikis and cancer

Apoptosis is a term coined to a process and phenomenon of programmed cell death or gene-regulated self-destruction. Its role is to shape the developing organism and maintain the homoeostasis of tissues and organs throughout mature life by limiting the absolute number of cells and maintaining their relative amounts (Meier et al., 2000; Danial and Korsmeyer, 2004). Excessive apoptosis causes tissue atrophy, as seen for instance in ischemic conditions. Deficient apoptosis results in proliferation or hyperplasia, and also contributes to cancer (Hanahan and Weinberg, 2000). Anoikis is a specific form of apoptosis induced by loss of cell adhesion or inappropriate cell adhesion (Stoker et al., 1968; Frisch and Francis, 1994, Meredith et al., 1993; Gilmore, 2005).

There are two main pathways, i.e. extrinsic and intrinsic, for mammalian cells to go into apoptosis (Hengartner, 2000). Permeabilization of the mitochondrial outer membrane (MOMP) is common to both the intrinsic and the extrinsic pathway, and is a decisive event in apoptosis. In MOMP, cytochrome C is released into the cytosol leading to activation of the caspase proteases. Caspases are present in inactive form in all cells, and they need activation to become functional (Timmer and Salvesen, 2007). MOMP is a rapid event and leads to an irreversible cascade of caspase activation and, eventually, to apoptosis (Goldstein et al., 2005; Upton et al., 2007).

The extrinsic pathway is activated by extracellular ligands binding to specific receptors at the cell surface called death receptors. These are molecules structurally similar to the tumor necrosis factor receptor (TNFR). This pathway activates a signaling cascade resulting in MOMP and later on to activation of caspases to their active form (Krammer, 2000).

The intrinsic pathway is activated by cellular stress (e.g. DNA damage, metabolic disorders) that results in loss of mitochondrial integrity (Bouchier-Hayes et al., 2005). Damage to the mitochondrial membranes leads to rupturing of the outer membrane and to the release of apoptogenic proteins (e.g. cytochrome c) into the cytosol (Bouchier-Hayes et al., 2005). Cytochrome c promotes the activation of caspases in the cytoplasm (Shi, 2001).

Typical features of apoptosis are: bulging of the cellular membranes outwards (blebbing) caused by breaking up of cytoskeleton, loss of the asymmetry of the lipid composition of the plasma membrane, and detachment of the cells from each other. The cell shrinks, the nucleus is fragmented, chromatin is condensed, and chromosomal DNA

becomes fragmented. Apoptosis differs from necrosis. In necrosis, the cellular debris from degrading cells induces inflammation and can harm the organism, whereas apoptosis does not induce inflammation at the site of cell death (Saraste and Pulkki, 2000).

Figure 2 gives an overview of apoptosis.

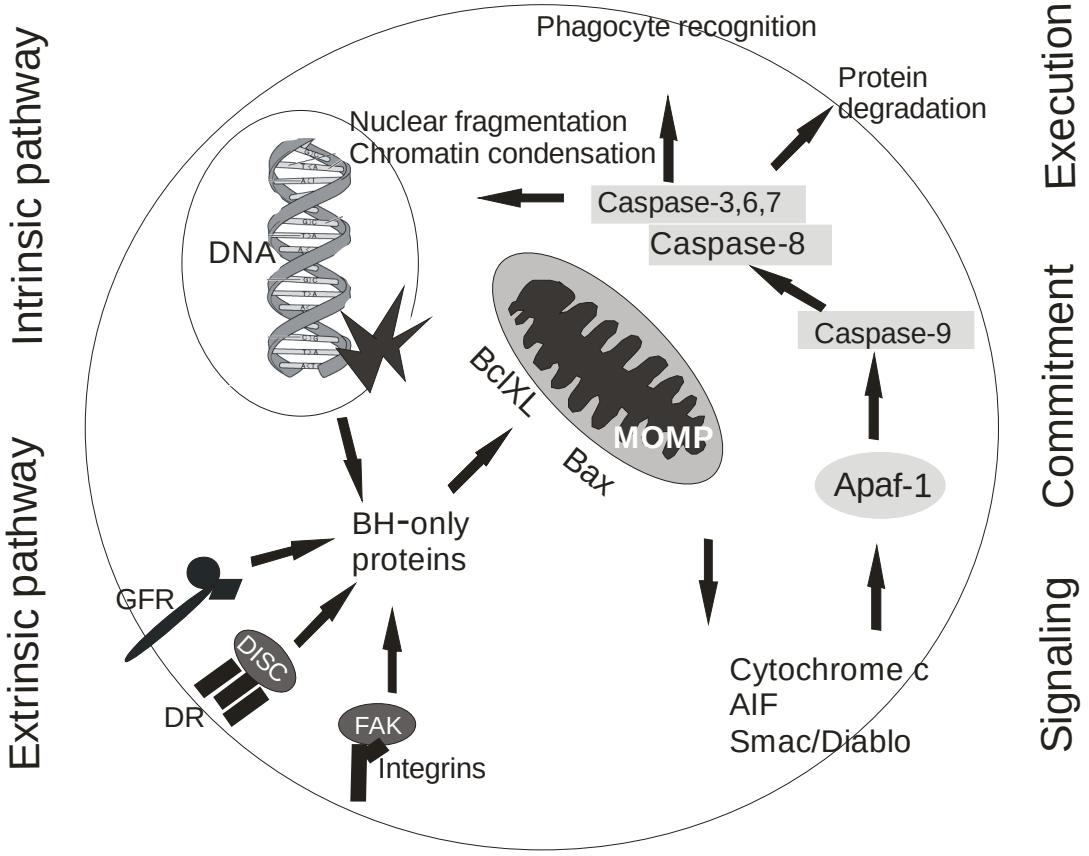


Figure 2 *Overview of apoptosis. Apoptosis can be triggered by various signals. It results in activation of BCL proteins and in permeabilization of the mitochondrial outer membrane. MOMP induces cleavage of caspases and apoptosis. (Modified from Valentijn et al., 2003).*

Apoptosis is regulated by two separate families of proteins, the Bcl-2 family and the proteins of the inhibitor of apoptosis (IAP) family (Chipuk et al., 2010). The Bcl-2-proteins are either pro- or antiapoptotic. The IAPs are anti-apoptotic (Cory and Adams, 2002; Mund et al., 2003). Figure 3 shows the classification of the Bcl-2 superfamily of proteins and IAPs by their functions.

The IAP family of proteins was originally described as inhibitors of caspases providing a cytoprotective step downstream of the death receptor or mitochondrial apoptosis. These proteins act by inhibiting cleavage of caspases to their active forms. IAPs also play a role in controlling mitosis and the cellular stress response. The role of survivin (one of the smallest members of IAP) in the regulation of cell fate is discussed later (Youle and Strasser, 2008; Altieri, 2006).

In order to survive, a normal cell requires attachment to the surrounding matrix via integrins (Valentijn et al., 2003). In normal epithelial cells, detachment from the matrix

leads to the recruitment of Bax and other BH3-proteins to the mitochondrial outer membrane; this eventually leads to apoptosis. Inadequate attachment leads to anoikis in normal cells (Frisch 1999; Wu et al., 2007). Cells can be rescued from apoptosis if they are reattached to the matrix, provided that MOMP has not been activated (Upton et al., 2007).

When epithelial cells are transformed with oncogenes such as v-src, v-Ha-Ras, treatment with phorbol esters, or exposure to migration-inducing factors such as hepatocyte growth factor (HGF), the cells lose their sensitivity to detachment-induced apoptosis (Frisch and Francis, 1994). In fact, survival of detached cells, commonly called anchorage-independent growth, is one of the hallmarks of cancer, as defined by Hanahan and Weinberg (2000).

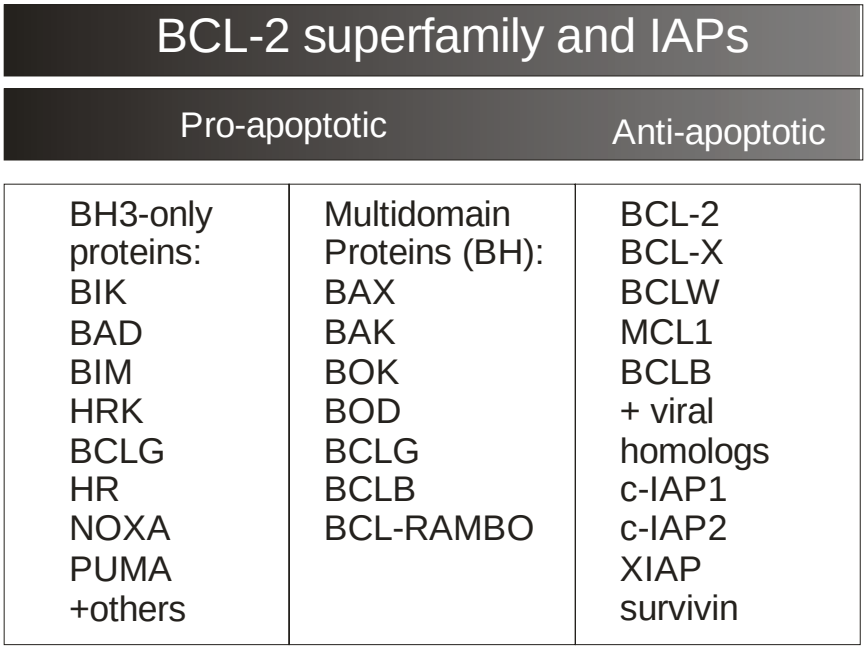


Figure 3 *List of members of BCL-2 superfamily and IAPs classified by their function as regulators of apoptosis and by their structural features. (Modified from Mayer, 2003).*

2.1.4 Energetics of normal and cancer cells

Cells produce energy to remain viable and to perform their genetically programmed functions. Energy is stored in adenosine triphosphate (ATP). It is derived mostly from oxidative phosphorylation in mitochondria, covering 88% of the energy produced. The other 12% is produced from substrate level phosphorylation through glycolysis in the cytoplasm and through the citric acid cycle, also known as the tricarboxylic acid (TCA) cycle, in the mitochondrial matrix (Veech et al., 2002). If the mitochondrial function is impaired, the cells adapt to the loss of energy by increasing glycolysis or glutaminolysis in order to maintain an adequate level of energy for viability (Donnelly and Scheffler, 1976; DeBerardinis, 2008).

Compared to normal cells, cancer cells produce more energy through substrate level phosphorylation. Prolonged reliance on substrate level phosphorylation for energy production brings about genome instability and cellular disorder (Szent-Gyorgyi, 1977; Warburg, 1956; 1931).

Cancer cells alter their metabolism. Aerobic glycolysis or the Warburg effect (shift of cell metabolism from oxidative phosphorylation and anaerobic glycolysis to aerobic glycolysis) is a robust metabolic feature of most tumors (Seyfried and Mukherjee, 2005; Semenza et al., 2001; Ristow, 2006; Gatenby and Gillies, 2004; Gogvadze et al., 2008). While there is no specific gene mutation or chromosomal abnormality common to all cancers, nearly all of them resort to aerobic glycolysis, regardless of their tissue or cell of origin (Loeb, 2001; Lengauer et al., 1998; Wokolorczyk et al., 2008; Nowell, 2002). Aerobic glycolysis involves elevated glucose uptake with lactic acid production in the presence of oxygen (Frezza and Gottlieb 2008; Gatenby and Gillies, 2007; Heiden Vander 2009). Genes for glycolysis are overexpressed in the majority of cancers studied (Ortega et al., 2009; Altenberg and Greulich, 2004). Only cells that are able to increase glycolysis during metabolic impairment are considered capable of developing into cancers (Warburg 1956).

On the other hand, mitochondria in cancer cells are structurally and functionally abnormal and incapable of generating normal levels of energy (Seyfried and Mukherjee, 2005; Chen et al., 2009; Ramanathan et al., 2005; John, 2001; Galluzzi et al., 2009; Foster et al., 1978). The most common abnormalities are reduced cardiolipin content and changes in the composition of the mitochondrial membranes leading to abnormalities in electron transport (Kiebish et al., 2008; Kocherginsky, 2009, Jiang et al., 2000; Claypool et al., 2008; Ohtsuka et al., 1993; Schug and Gottlieb, 2009). The function of cardiolipin is to prevent oxidation of the coenzyme Q couple by preventing the formation of reactive oxygen species (ROS). ROS can be harmful to DNA (Kiebish et al., 2008; Veech, 2004). Increased production of ROS impairs the stability of the genome, tumor suppressor gene function, and thereby control of cell proliferation and cell survival (Loeb, 2001; Trachootham et al., 2009).

Under normal conditions, damage to mitochondrial function induces apoptosis. Cancer cells, however, display increased resistance to apoptosis-inducing permeabilization of the mitochondrial membranes (Kroemer and Pouyssegur, 2008). On the other hand, up-regulation of antiapoptotic genes in cancer cells interferes with the apoptotic signaling cascade. This acts in the direction of preventing programmed cell death (Holley and St Clair, 2009).

2.2 Selected cancer-related signaling pathways

The activity of signaling pathways is typically altered in cancer. The introduced signaling pathways are affected by src-transformation in MDCK cells.

2.2.1 Src tyrosine kinases

Src was the first oncogene found. It is a nonreceptor protein tyrosine kinase. Viral Src (v-Src) is a mutated and constitutively active form of a normal cellular src (c-src). As a proto-oncogene, src displays intrinsic tyrosine kinase activity (Collett and Erikson, 1978; Levinson et al., 1978). Its tyrosine kinase activity is regulated by the phosphorylation of its tyrosine (Y) residue 416 in the C-terminus of the molecule (Thomas and Brugge, 1997). V-Src has lost the residues responsible for the regulation of kinase activity due to mutational changes in the C-terminus and, thus, it is constitutively active. As signaling proteins, c- and v-Src kinases phosphorylate their protein substrates exclusively on tyrosyl residues (Hunter and Sefton, 1980).

In epithelial cells, c-Src is associated with adherens junctions and focal adhesions, pointing to its role in cell-cell and cell-matrix adhesion (Tsukita et al., 1991; Frame et al., 2002). c-Src activation is found in carcinoma cell migration and metastasis (Sakamoto et al., 2001).

Src has several target proteins. Most of them are located at focal adhesions and are key components in integrin-mediated signal transduction and in actin-binding protein complexes. They include junctional proteins α- and β-catenin, ZO-1, occludin, p120-catenin (p120ctn), connexin 43, nectin-2δ (Parsons and Parsons, 1997; Thomas and Brugge, 1997; Tsukamoto and Nigam, 1999; Abram and Courtneidge, 2000; Kikyo et al., 2000). Other targets include enzymes involved in phospholipid metabolism, such as PLC-γ, p85 subunit of PI3-kinase. Moreover, signaling proteins such as p190RhoGAP, p120rasGAP, and epithelial growth factor receptor (EGFR) substrate Eps8 also serve as targets of Src (Thomas and Brugge, 1997; Abram and Courtneidge, 2000; Gallo et al., 1997). Focal adhesion kinase (FAK) is an upstream activator of src (Thomas and Brugge, 1997).

Although src is found in the structures associated with cell-matrix and cell-cell adhesion, its precise role in cell adhesion is still poorly understood. Src is not essential for the formation of focal adhesions, as shown in studies utilizing Src-/- fibroblasts. It is nevertheless needed for the optimal adhesion and spreading of fibroblasts on fibronectin matrix via integrin receptors (Felsenfeld et al., 1999; Cary et al., 2002).

In MDCK cells, the lowered level of expression of v-Src is associated with derangement of the formation of adherens junctions. On the other hand, it has no effect on the assembly of tight junctions and desmosomes (Warren and Nelson, 1987). In v-src transformed MDCK cells, an elevated level of src activity is accompanied by weakening of the cadherin-based adherens junctions (Volberg et al., 1992; Behrens et al., 1993; Takeda and Tsukita, 1995). It is not known whether v-Src or c-Src interact directly with the components of adherens junctions *in vivo* (Takeda and Tsukita, 1995).

2.2.2 Focal adhesion kinase

Focal adhesion kinase is an activable kinase and a central signaling element in focal adhesions (FA). It regulates apoptosis by canonical or non-canonical pathways. The

canonical FAK pathway prevents apoptosis via the PI3-kinase/PKB and MAPK pathways (Mitra and Schlaepfer, 2006). The non-canonical FAK pathway regulates apoptosis via p53 or death receptor signaling (Frisch, 1999; Wu et al., 2007).

In malignant cells, such as mammary tumor cells, FAK acts by suppressing anoikis and increasing tumor cell survival in the blood stream, and by enhancing lung colonization (Mitra and Schlaepfer, 2006). In tumors, the over activation of FAK induces metastasis (Ewings et al., 2007; Dehan et al., 2009). Thus, FAK is an important component in the evasion of anoikis in malignant cells (Burridge et al., 1998; Mitra and Schlaepfer, 2006).

2.2.3 Ras and Rho GTPases

Ras was the first cellular oncogene (c-onc) found in human tumor cells (Der et al., 1982). Since then, it has been thoroughly studied and found to be present in various human cancers (Barbacid, 1987, Hamad et al., 2002). It also occurs as a viral oncogene v-Ras (Rasheed et al., 1983). The Ras proteins are GTP-binding proteins that act as molecular switches in cell signaling. They mediate signals for example from growth factor receptors to various effector proteins in several signaling pathways. Ras proteins are typically activated by activated receptor tyrosine kinases (RTK) or G protein-coupled receptors (Vojtek and Der, 1998). The EGFR signaling pathway is one of the pathways utilizing Ras as its downstream effector. It can induce mitosis, apoptosis, proliferation, oncogenic transformation, enhanced motility, protein secretion, and differentiation or dedifferentiation, depending on the cell type and its environment (Seger and Krebs, 1995).

Small GTPases of the Rho family are among the proteins that regulate cadherin-mediated cell-cell adhesion (Anastasiadis and Reynolds, 2000; Noren et al., 2000). The effects of small GTPases depend on the cell type; the same signal may lead to different outcomes in different cell types (Sahai and Marshall, 2002). Active GTPases affect the function and morphology of cell-cell junctions and the localization of tight junctional components, i.e. occludin and ZO-1 (Jou et al., 1998).

2.2.4 Phosphatidyl-inositol 3 kinase pathway

Phosphoinositide 3-kinases (PI3K) are another important family of signaling proteins downstream of many growth factor receptors and Ras. The binding of PI3K either to the activated growth factor receptor or to Ras brings PI3K in contact with the plasma membrane where it becomes activated and acts on its lipid substrates (Cantley, 2002).

The major target of PI3K is phosphatidylinositol (4, 5)-bisphosphate (PIP2) which is converted into phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) through the phosphorylation of the hydroxyl groups in the inositol head group. PIP3 serves as a docking site for the serine-threonine kinase Akt, also known as protein kinase B (PKB). The main effect of Akt/PKB is the enhancement of cell survival by inhibiting apoptosis and stimulating cell proliferation and cell growth via its effector molecules (Cantley, 2002; Engelman et al., 2006).

The activity of Akt/PKB is regulated by the levels of PIP3. It varies, depending on whether PI3K is in active state due to a mitogenic signal or not. Elevation of PIP3 is counteracted by the activity of PI3K phosphatases, which remove the 3' phosphate group from PIP3. The best known such phosphatase is PTEN. The level of PTEN is controlled at the transcriptional level by PI3K (Vasudevan et al., 2004; Han et al., 2008).

Three separate mechanisms in human cancer can lead to dysregulation of the PI3K-Akt/PKB pathway: hyperactivity of PI3K, hyperactivity of Akt/PKB, or inactivity of PTEN. In fact, mutated forms and amplifications of PI3K class IA and activating mutations of Akt/PKB are found in a variety of cancers (Samuels et al., 2004). PI3K is hyperactive in almost 30% of human colon carcinomas. Constitutively active Akt1 promotes aberrant growth of prostate cells in a transgenic mouse model (Majumder et al., 2003).

The most important genetic derangement in the PI3K/Akt/PKB pathways is the loss of PTEN function. Its prevalence in all human cancers has been estimated to be 40%. This is in line with the fact that PTEN was originally discovered as a tumor suppressor gene, absent in various cancers (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997; Maehama and Dixon, 1998; Stambolic et al., 1998).

2.2.5 Wnt signaling

Wnt factors are extracellular matrix- and cell-membrane-associated growth factor proteins that act on Frizzled (Frz) receptors. They have an important role in directing cell proliferation and establishing and determining cell polarity and cell fate during embryonic development (Logan and Nusse, 2004). Consequently, mutations in the Wnt pathway are often linked to human birth defects, cancer, and other diseases (Clevers, 2006).

β-catenin has a dual role as a transcriptional activator and an adhesion molecule. It is the most important target protein of the canonical Wnt pathway. In the absence of Wnt, β-catenin is constantly degraded by the actions of the Axin complex (Seifert and Mlodzik, 2007; Wang and Nathans, 2007). The Axin complex is composed of the scaffolding protein Axin, APC, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). In the absence of wnt, CK1 and GSK3 phosphorylate the amino terminal region of β-catenin. This then leads to ubiquitination of β-catenin and, subsequently, to its proteasomal degradation (He et al., 2004). The elimination of β-catenin prevents it from reaching the nucleus, binding to a transcription factor TCF/LEF, and from activating the wnt signaling target genes. In the presence of Wnt, Frz is activated, leading to dissolution of the Axin complex. This leaves β-catenin in an unphosphorylated state which prevents it from degradation. This in turn leads to the accumulation of β-catenin in the cytoplasm and enables it to travel to the nucleus. There it forms a complex with TCF/LEF and serves as a co-activator to wnt-responsive genes (MacDonald et al., 2009).

There is ample evidence of the role of deregulated Wnt/β-catenin signaling in cancer. Especially in colon cancer, APC deficiency or β-catenin mutations prevent proteosomal degradation of β-catenin, which then leads to constitutively active β-catenin signaling (Polakis, 2007).

Among the genes upregulated by β-catenin/LEF complex is a well-established anti-apoptotic protein survivin. Due to the circuitry of the β-catenin/LEF signaling pathway described above, an inverse relationship exists between the integrity of the cadherin-based junctional complexes, so that cells with intact junctions and with β-catenin bound to E-cadherin do not express survivin. In cancer cells, on the other hand, in which β-catenin accumulates in the nucleus, there is upregulation of survivin with its anti-apoptotic effects. This may be one of the mechanisms underlying the role of E-cadherin as a tumor suppressor (Iurlaro et al., 2004; Torres et al., 2008; Syed et al., 2008).

2.2.6 Transforming growth factor β

Transforming growth factor β (TGFβ) belongs to a family of dimeric polypeptide growth factors that regulate embryonal development and tumorigenesis (Kuzuya and Kinsella, 1994). TGFβ regulates negatively cell proliferation, promotes differentiation, induces apoptosis, and takes part in wound healing, angiogenesis and embryonic development (Kuzuya and Kinsella, 1994). TGFβ acts as a tumor suppressor in normal cells by inhibiting proliferation or by driving the cells towards differentiation or apoptosis (Blobe et al., 2000). In addition, TGFβ has been suggested to be an important differentiation-inducing component in Matrigel, a growth substratum used commonly in cell culture studies (Kuzuya and Kinsella, 1994).

Advanced cancer and metastatic tumors overexpress TGFβ. Malignant cells lose their TGFβ-mediated growth inhibition in the early stages of cancer. This may be caused by a mutation or loss of expression of genes encoding proteins of the TGFβ signaling pathway (Oft et al., 1998).

2.2.7 Survivin

Survivin is the smallest member of the inhibition of apoptosis family proteins (IAP). It is not present in mature tissues, but can be found in various cancers (Li and Altieri, 1999; Altieri, 2006). Survivin has a dual role in inhibiting apoptosis and promoting proliferation. Its cytosolic localization appears to be associated with its function as an inhibitor of apoptosis, whereas nuclear localization seems to correlate with its role in cell division and proliferation (Altieri, 2006).

Survivin regulates apoptosis by several mechanisms: first, by opposing mitochondrial dysfunction and, secondly, by antagonizing caspase activity (Lu et al., 2003). In addition, it regulates the integrity of the mitotic apparatus (Jeyaprakash et al., 2007). The expression of survivin is increased at the G2/M interphase. It protects cells against possible default induction of apoptosis in the case of aberrant mitosis (Li et al., 1998). Survivin is associated with Aurora-B kinase at the chromosome passenger complex that segregates the chromosomes and has a role in cytokinesis (Skoufias et al., 2000). It is required for the maintenance of the spindle assembly checkpoint that monitors the proper alignment of

microtubules and, in this way, secures the fidelity of cell division (Lens et al., 2003). Lack of survivin causes polyploidy as well as apoptosis (Yang et al., 2004).

Survivin has been found in every human tumor studied, including those with a low mitotic index (Fukuda and Pelus, 2006). Cytoplasmic and nuclear survivin is present in invasive cancers, and is a sign of poor prognosis. Survivin is also associated with metastasis and with enhanced cell survival (Qi et al., 2010; Mehrotra et al., 2010). Functional loss of wild type p53 is often associated with upregulation of survivin (Altieri, 2006).

No mutations or polymorphisms have been identified that would selectively contribute to survivin gene transcription in tumors. However, alterations are common in the proteins regulating survivin expression (Altieri 2006).

Figure 4 shows the complex network of regulatory factors affecting the expression of survivin. Expression of tumor suppressors such as p53, PTEN and FOXO inhibit the expression of survivin. On the other hand, the presence of some oncogenes such as PI3K, AKT and TCF-4/β-catenin signaling enhances the expression of survivin. Survivin acts as a regulatory molecule in determining cell fate (Guha and Altieri, 2009).

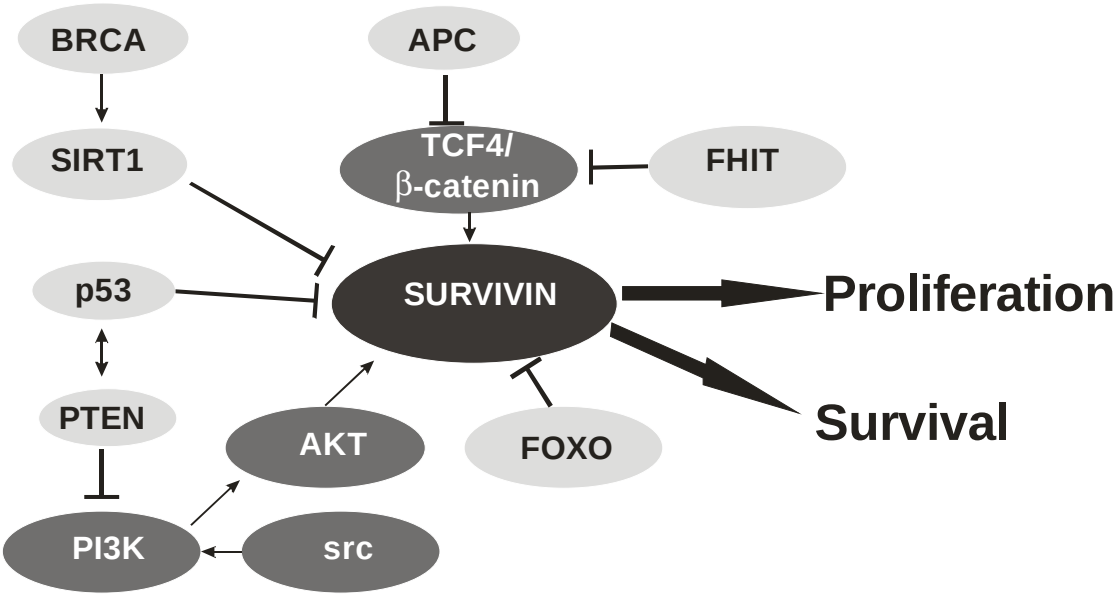


Figure 4 *Oncogene and tumor suppressor networks targeting the survivin pathway. (Modified from Guha and Altieri, 2009).*

2.3 Structural and functional properties of epithelial cells

Epithelial cells delineate organs and glands. Epithelium can be divided functionally and structurally into barrier, absorptive, secretory, and sensory epithelium. In order to maintain its proper functions, correct polarization of epithelium is needed (Rodriguez-Boulan and Nelson, 1989; Matlin and Caplan, 1992; Rodriguez-Boulan and Powell, 1992).

An actin cytoskeleton, a spectrin-based membrane-skeleton and microtubules are needed to maintain the polarized phenotype (Mays et al., 1994). Spectrin (also known as fodrin) is a cytoskeletal protein which, along with other linking proteins at the intracellular face of the plasma membrane, forms a scaffolding structure known as the membrane skeleton of the plasma membrane. Spectrin forms a hexagonal web that is associated with short actin oligomeres anchoring the actin cytoskeleton to the membrane skeleton. It takes part in establishing and maintaining cell polarity, cell shape, and proper localization of several transmembrane proteins, such as some receptors, in the plasma membrane. In most polarized epithelial cells, the spectrin is localized along the lateral cell walls (Nelson and Veshnock, 1987).

Other principal components of the polarity are the cell-cell junctions. They include tight junctions (TJ), gap junctions, adherens junctions (AJ) and desmosomes (D). Tight junctions have a role in the formation of lateral cell walls, and in forming a barrier restricting diffusion of lipids between the apical and lateral plasma membranes. A polarized phenotype is important for epithelial cells, e.g. in enabling the vectorial transport of molecules through the epithelium (Rodriguez-Boulan and Nelson, 1989; Mays et al., 1994; Brown and Stow, 1996).

The plasma membrane of epithelial cells is divided into two faces: an apical surface facing the lumen, on the one hand, and a basolateral surface apposing adjacent cells and the underlying ECM, on the other hand (Mostov, 2003; Rodriguez-Boulan and Nelson, 1989; Brown and Stow, 1996). In most epithelial cells, the apical and basolateral surfaces have distinct functions. This is reflected in their distinct protein and lipid compositions. The apical membrane is highly enriched in glycosphingolipids. The basolateral membrane, on the other hand, has a higher concentration of phosphatidyl choline and sphingomyelin (Simons and van Meer, 1988).

In addition to the overall differences in the lipid composition between the various domains, there is also asymmetry in the lipid content of the two leaflets, so that the inner leaflet is rich in phosphoinositides, a specific class of lipids that contain phosphatidylinositol and its phosphorylated derivatives. They are involved in many critical events of cellular physiology, such as regulation of membrane trafficking, intracellular signaling, cytoskeleton organization, and apoptosis (Di Paolo and De Camilli, 2006). For instance, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2, PIP2) is characteristic of the apical surface, and phosphotidylinositol 3,4,5 trisphosphate (PtdIns (3,4,5)P3, PIP3) of the basolateral surface in mammalian epithelial cells (Martin-Belmonte et al., 2007; Gassama-Diagne et al., 2006).

Microvilli and cilia are specialized membrane structures typical of the apical or lumen-facing side of epithelial cells. Transmembrane proteins, i.e. integrins, are characteristic of the basal domains. Integrins interact with the basal lamina proteins – laminin, collagen IV and heparan sulfate proteoglycans (Rodriguez-Boulan and Nelson, 1989; Matlin and Caplan, 1992; Brown and Stow, 1996).

Epithelial cells execute their predetermined polarity program to produce epithelium. The polarized phenotype is characterized by strictly determined localization, organization, and functioning of cellular organelles and cytoskeletal structures, as well as by vectorial

functions such as directional transport of molecules (Rodriguez-Boulan and Nelson, 1989, Gumbiner, 1992, Mays et al., 1994).

2.3.1 Cell-cell junctions

Cell-cell junctions

The cell-cell junctions in epithelial cells are divided into three groups: 1) communicating junctions, such as gap junctions (Bennett et al., 1991), 2); anchoring junctions, such as desmosomes, and adherens junctions (Steinert and Roop, 1988, Tsukita et al., 1992; Hartsock and Nelson, 2008); and 3) sealing junctions, such as zonula occludens or tight junctions (Schneeberger and Lynch, 1992; Anderson et al., 1993). The junctional proteins provide a link to the actin cytoskeleton. They also serve as focal nodes or scaffolding sites to which various signaling proteins link, thus forming multi-molecular signaling complexes. The protein complexes characteristic of these structures are dynamic, and their composition depends on the integrity and maturity of the junctions (Feigin and Muthuswamy, 2009).

Figure 5 is a schematic illustration of a polarized cell and the localization of the cell-cell and cell-matrix junctions along the cell walls.

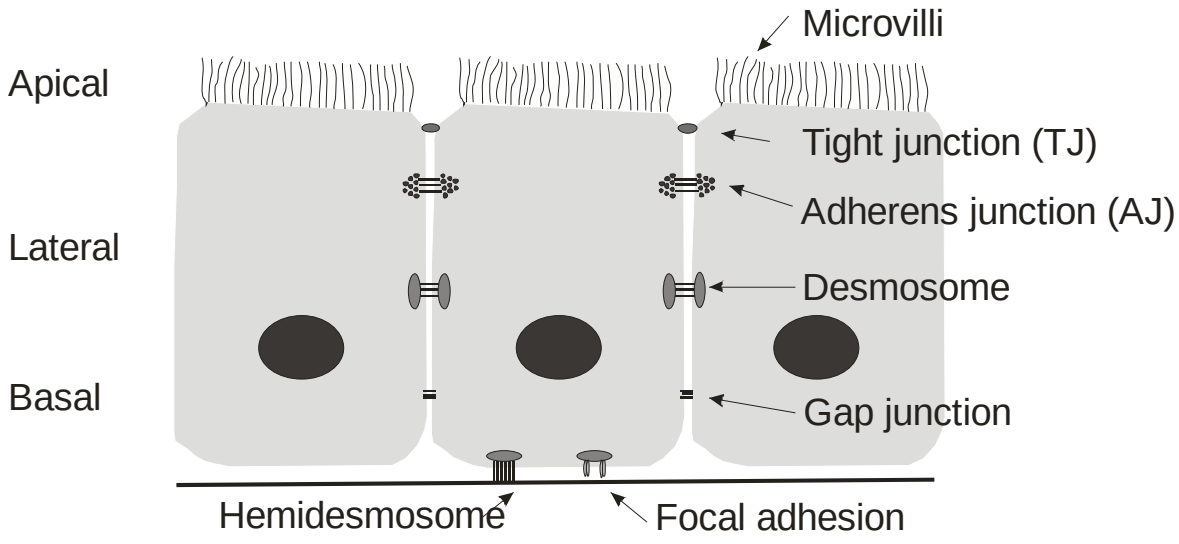


Figure 5 *A schematic illustration of cell-cell junctions of a polarized epithelial cell. The basal side faces the growth substratum, and the cells attach via focal adhesions or focal contacts and hemidesmosomes. The cells attach to each other via gap junctions, adherens junctions and desmosomes, and the apical side is sealed with tight junctions.*

Cadherin-based adherens junctions

Cadherins are the principal transmembrane proteins of cell-cell adherens junctions (AJ). They are Ca^{2+}-dependent and bind to actin filaments via cytoplasmic catenins (Takeichi, 1995). Cadherins form the physical basis of cell-cell adhesion, they regulate cell shape, detachment, migration, proliferation, and differentiation (Fagotto and Gumbiner, 1996; Gooding et al., 2004).

Cadherins bind via β-catenin to γ-catenin (plakoglobin) and to α-catenin and further on to actin filaments, α-actinin, vinculin, ZO-1, spectrin, and a number of other molecules at adherens junctions. The complex stabilizes the structure of adherens junctions (Yamada and Geiger, 1997; Kemler, 1993). The interactions between E-cadherin-, β-catenin-, α-catenin- and actin filaments at the region of cell-cell contacts are dynamic and enable the cell to respond rapidly to various stimuli (Drees et al., 2005; Yamada et al., 2005).

E-cadherin is considered to be a tumor suppressor protein. The expression of E-cadherin has been observed to decrease or disappear in many human carcinomas (Hirohashi, 1998; Berx and van Roy, 2001). Loss of E-cadherin weakens cell-cell junctions, and loss of its expression or function is associated with cell invasion in carcinomas (Behrens et al., 1993). On the other hand, forced expression of exogenous E-cadherin in transformed epithelial cell lines reduces their invasive potential and restores the normal phenotype (Cavallaro and Christofori, 2004).

Cadherins modify cell signaling directly by regulating the organization of signaling components, or indirectly by the formation of cell-cell contacts (Fagotto and Gumbiner, 1996). An altered state of the cell-cell contacts, in turn, regulates cell growth and gene expression. Also other components of adherens junctions have a dual role as structural proteins, and as cell signaling proteins, such as β-catenin and p120 cas. They regulate the localization and, thus, the function of cadherins (Tsukita et al., 1991; Woods and Bryant, 1993; Brady-Kalnay et al., 1995). Cadherin seems to be indispensable for the normal growth and polarization of epithelial tissue, presumably due to its organizing functions (Fagotto and Gumbiner, 1996).

β-catenin has a dual role in binding E-cadherin to the cytoskeleton and in taking part in the Wnt-signaling pathway. Wnts are extracellular signaling molecules encoded by ‘wingless’ genes. (Behrens et al., 1996; Huber et al., 1996; He et al., 1998; Shtutman et al., 1999; Tetsu et al., 1999; Shimokawa et al., 2003). The role of β-catenin, wnt-signaling, and cancer is discussed later in section 2.4.4.

Cell-cell adhesive and transcriptional functions of β-catenin are tightly bound together. β-catenin is involved in cancer cell morphology and motility, and it has an important role in different stages of carcinogenesis (Gavert and Ben-Ze'ev, 2007; Brembeck et al., 2006).

Tight junctions and ZO-1

Tight junctions are the most apical intercellular junctions in epithelial cells. They serve as selective diffusion barriers sealing the space between adjacent cells. They also maintain the different lipid and protein composition in the apical and basolateral plasma membrane

domains (fence function). Tight junctions also have a role, together with adherens junctions, in regulating the growth and differentiation of the cells (Balda and Matter, 1998; Tsukita et al., 1999).

ZO-1 was the first tight junctional protein identified (Stevenson et al., 1986). It is a cytoskeletal protein, but plays also a part in cell signaling and in regulating proliferation (Georgiadis et al., 2010). In epithelial cells, ZO-1 initiates the formation of cell-cell junctions. It, together with E-cadherin, accumulates at the edges of the cellular protrusions of the adjacent cells, forming spot-like junctions (Yonemura et al., 1995). In the early stages of polarization, ZO-1 remains bound at tight junctions, whereas E-cadherin is released from the complex to form adherens junctions (Ando-Akatsuka et al., 1999).

Gap junctions

Gap junctions are intercellular structures joining together the cytoplasm of neighboring cells. The hemichannels are composed of connexins (Beyer et al., 1990). They enable the passive diffusion of ions and small molecules between cells (Kumar and Gilula, 1996). Most cells communicate via gap junctions (Saez et al., 1993).

Desmosomes

Desmosomes are specialized junctional structures that form a tight connection between adjacent epithelial cells (Schwartz et al., 1990). The desmosomal structure consists of several transmembrane adhesive glycoproteins, such as desmoglein and desmocollin and cytoplasmic plaque proteins. The desmosomal proteins are linked to cytokeratins (Garrod, 1993).

2.3.2 Cell-substratum contacts

Integrins

Integrins are a superfamily of transmembrane αβ-heterodimeric cell adhesion receptors located at focal adhesion sites and hemidesmosomes. They link the actin cytoskeleton to the extracellular matrix (ECM) ligands. Integrins sense tension and can modulate the actin cytoskeleton. On the other hand, binding to the matrix can modulate the functional state and other interactions of integrin. Thus, engagement of integrins to their cognizant extracellular matrix ligands initiates a variety of signal transduction pathways which affect adhesion, proliferation, survival or apoptosis, cell shape, polarity, motility, gene expression, and differentiation (Hynes, 2002).

Figure 6 depicts schematically the membrane orientation and the structure of the integrin heterodimer.

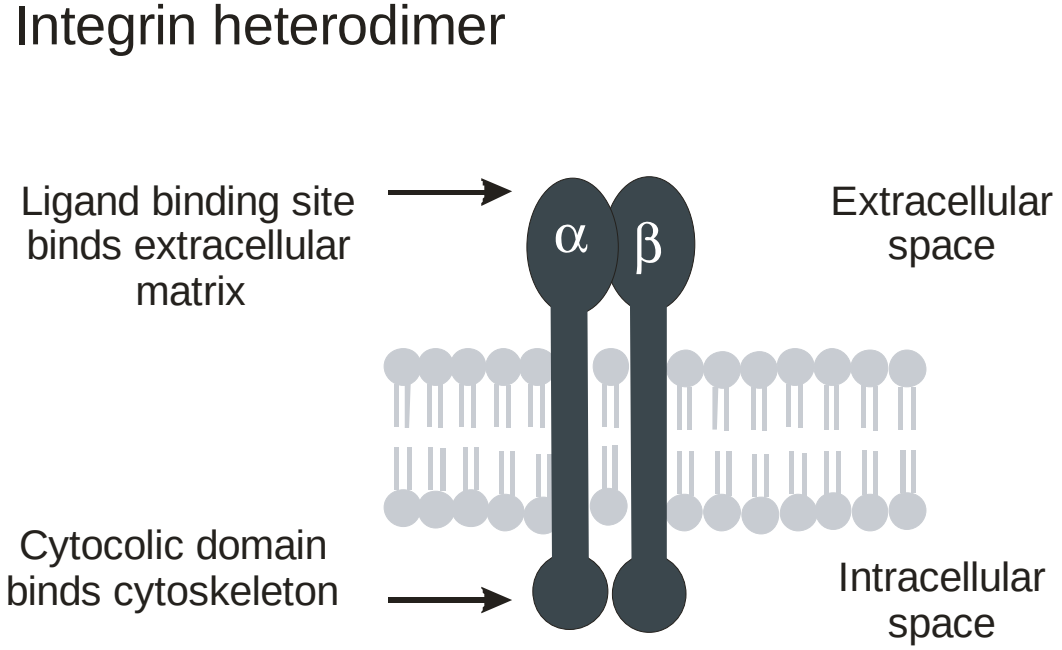


Figure 6 *Structure of integrins (modified from Hynes, 2002).*

Most stationary cells adhere to their underlying substratum via cell-matrix junctions, or focal adhesion plaques, focal contacts or focal adhesions (FA) (Abercrombie et al., 1971; Burridge et al., 1988). The major molecules in FAs are transmembrane proteins, integrins that join the ECM and cellular cytoskeleton. Integrins connect the extracellular proteins to the actin cytoskeleton. The first step in the formation of the FA is ligation of integrins to their specific matrix anchors (Yamada and Miyamoto, 1995). Various regulatory proteins, such as calcium-dependent protease calpain II, protein kinase C, FAK and Src family tyrosine kinases control the assembly of focal adhesions (Beckerle et al., 1987; Jaken et al., 1989; Burridge and Chrzanowska-Wodnicka, 1996; Kaplan et al., 1994; Zamir and Geiger, 2001). Signaling via FAs is important for cell survival (Burridge et al., 1998). Table 1 shows some of the principal components of focal adhesions.

Components of focal adhesions	**Function**
Integrins	Membrane-associated
Syndecans	Membrane-associated
Talin	Cytoskeletal
Paxillin	Cytoskeletal
Tensin	Cytoskeletal
Vinculin	Cytoskeletal
Dynamin	Cytoskeletal
VASP	Signaling
c-src	Signaling
Rho GTPases	Signaling
FAK	Signaling

Table 1 *The principal components of focal adhesions.*

Hemidesmosomes consist of multimeric protein complexes that link epithelial cells to their underlying matrix, and that serve as cell surface anchorage sites for the keratin cytoskeleton. They are localized at the basal surface. The major components of hemidesmosomes are plectin, keratins, laminin, and α6β4-integrin (Schwartz et al., 1990; Garrod, 1993).

2.3.3 Regulation of epithelial cell polarity and formation of the lumen

The key event in the polarization of epithelial cells is the establishment of cell-cell junctions. Building of the polarized epithelium utilizes three separate, interacting machineries that are collectively called the epithelial polarity program (Tanos and Rodriguez-Boulan, 2008).

The first component is the vesicular trafficking machinery, which acts via its secretory and endocytic functions that deliver plasma membrane proteins and lipids to the apical and basolateral PM domains. Second, the domain-identity machinery targets the proteins and lipids to their destinations by recognizing and binding to specific plasma membrane domains. Third, tight junctions form a barrier between the apical and basolateral domains. The small GTPases play a pivotal role in controlling cell growth and cytoskeleton organization by establishing the polarized epithelial cell morphology (Tanos and Rodriguez-Boulan, 2008).

Many epithelial tissues have a glandular, tubular or sphere-like architecture, in which the epithelial cells form a single cell-thick peripheral lining layer with a central lumen. In the lining epithelium, the apical faces of the cells are oriented towards the lumen, while the basal membranes face towards the surrounding matrix. In *in vitro* studies, the equivalent of the tubular or glandular epithelial structures is a sphere or a cyst. In 3D cell cultures, some epithelial cells form a sphere with a hollow center in which the apical side of the cells within the cortical layer faces the lumen. In such a cyst-like formation, the cells are attached to each other along the lateral walls and to the substratum along their basal surface (Moreno-Bueno, 2003). It is not clear how epithelial cells form an apical lumen, but the initial signal comes from the basally located integrins that recognize and bind to the ECM (O'Brien et al., 2001). For instance, in some cells the interaction of collagen I with β1-integrin triggers a signaling pathway that orientates the apical side of the cyst (O'Brien et al., 2001; Yu et al., 2005).

In vitro studies have demonstrated that small GTPases and PTEN are important in the polarization and lumen formation of epithelial cells (O'Brien, 2001). During the lumen formation, PTEN is targeted to the apical membranes where it mediates the separation of PIP2 to the apical, and PIP3 to the basolateral surface. Cdc42, a member of the Rho family of GTP binding proteins, plays a role in the formation of polarized actin structures. It is instrumental in the establishment of the subapical actin cytoskeleton, the apical surface and the lumen (Martin-Belmonte, 2007). Also Rac1 has an important role in polarity – the inhibition of Rac1 or β1-integrin leads to inversion of polarity and abnormal lumen formation (O'Brien et al., 2001; Yu et al., 2005).

Annexin 2 (Anx2) is a Ca^{2+}-dependent membrane and F-actin-binding protein. Its intracellular location is at the sites of membrane rafts. In the rafts, Anx2 may function as an organizer of these membrane microdomains and their connection to the actin cytoskeleton and formation of the actin cytoskeleton. Anx2 is recruited, along with Cdc42, to the apical plasma membrane (Plant et al., 2000).

Some 3D cell culture studies on lumen formation in MDCK cells have suggested that the formation of the apical surface and lumen is mediated by exocytosis of vacuolar apical compartments (VAC). The VACs are cytoplasmic vacuoles that contain in their membranes several components of apical membranes. In some cell types, such as MDCK cells, they are rapidly transported to the apical membrane, thus contributing to its expansion (O'Brien et al., 2002; Vega-Salas et al., 1987; Martin-Belmonte and Mostov, 2007). Down-regulation of Cdc42 or expression of its dominant negative mutants result in abnormal lumen formation and accumulation of intracellular lumens via the coalescence of VACs (Martin-Belmonte and Mostov, 2007).

2.4 Epithelial-mesenchymal transition

In epithelial-mesenchymal transition (EMT), the epithelial cells lose their characteristic features and acquire a mesenchymal phenotype and become more motile (Hay, 1995). Loss of epithelial junctions and changes in actin cytoskeleton are phenotypic to EMT (Thiery, 2002). Several factors can induce EMT (Huber et al., 2005). It is a normal feature in the embryonic development of tissues and organs, but it can be transient in cancer progression, and permanent in highly invasive tumors with a mixed carcinoma/sarcoma appearance (Halbleib and Nelson, 2006; Jeanes et al., 2008). EMT enables cancer cells to invade and metastasize (Huber et al., 2005). The alterations in polarity are difficult to identify in carcinomas *in situ*, but are clearly seen in invasive carcinomas with sarcomatous characteristics (Halbleib and Nelson 2006).

E-cadherin plays an important role in EMT. Its expression is decreased upon EMT. Mutations in E-cadherin or in catenins leading to EMT are typical of metastatic carcinomas (Thiery, 2002; Moreno-Bueno et al., 2008). Many oncogenes seem to have a role in EMT, e.g. they contribute to the disassembly of junctional complexes and promote proliferation (Tanos and Rodriguez-Boulan 2008). The EMT-associated disassembly of cell-cell contacts correlates with tumor grade and stage (Benjamin and Nelson, 2008). However, mere repression of E-cadherin expression is not sufficient for EMT. Additionally, changes that directly enhance the ability of cells to migrate and their capacity to secrete ECM-digesting proteases are required (Kalluri and Weinberg, 2009).

In EMT, the apical-basolateral polarity of epithelial cells is replaced by antero-posterior polarity. In the epithelial phenotype, the apical side of the cells faces the cell culture medium or the lumen, and the cells are attached to each other laterally and to the cell culture dish or the ECM basally. In the mesenchymal phenotype, which develops when cells undergo EMT, the cells acquire a spindle-like phenotype. Their vertical polarity is replaced by horizontal polarity, so that the leading or the moving edge is the anterior side, and the other side of the cell is called the posterior side (Condeelis et al.,

2005). Cell signaling and gene expression are altered in EMT. For instance, cells lose their epithelial markers and start expressing proteins typical to mesenchymal cells (Thiery et al., 2002).

In Figure 7 the major morphological changes that take place in EMT are shown schematically, as observed in cells in monolayer (2D) cultures. Also the major epithelial markers replaced by mesenchymal ones appearing upon EMT are indicated (Kalluri and Weinberg, 2009).

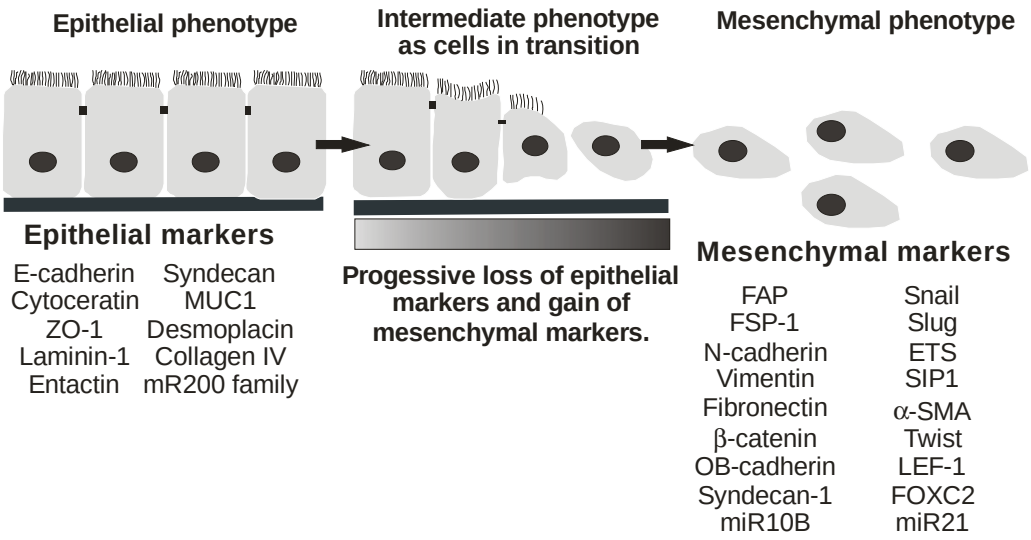


Figure 7 *EMT in 2D. Disruption of cell-cell adhesion and change of cell morphology from cuboidic epithelial cells to elongated non-polarized cells are phenotypic to EMT in 2D. The cells become more motile. The epithelial markers are replaced by mesenchymal ones in EMT. (Modified from Kalluri and Weinberg, 2009)*

The morphological changes in EMT can be seen in microscopic observation of the monolayer cultures. Different markers can also be used to detect the status and phase of EMT. For instance, E-cadherin and ZO-1 are commonly used markers for polarized epithelium; N-cadherin and vimentin can be used as mesenchymal markers (Kalluri and Weinberg, 2009).

Monolayer cultures can also be used to assess the motility of cells undergoing EMT by using the wound-healing migration assay, in which a needle is used to make a wound in a dense monolayer of epithelial cells. This induces migration of cells from the wound edges. These cells have all the characteristics of cells undergoing EMT. They acquire a migratory phenotype and move into and fill the empty space in the wound area (Kalluri and Weinberg, 2009).

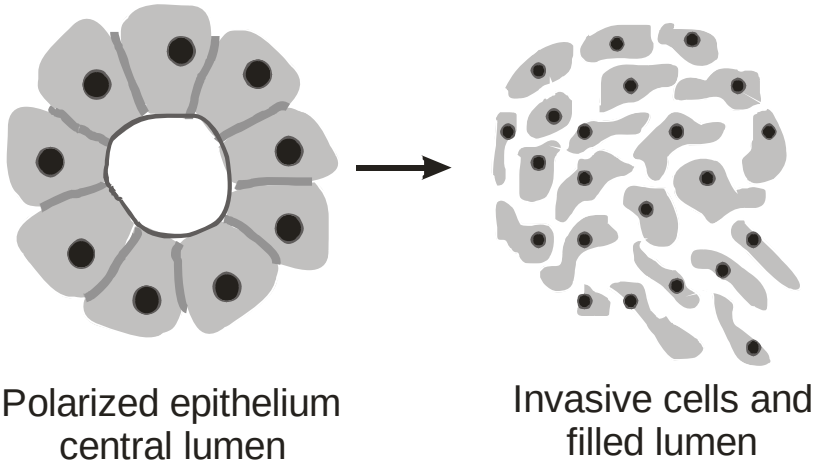


Figure 8 *EMT in 3D. Disruption of cell adhesion and polarity is phenotypic to EMT in 3D. The cells become more elongated and are able to invade the surrounding matrix. Filling of the lumen can be seen in glandular tissues. (Modified from Kalluri and Weinber, 2009).*

3D cultures allow an experimental setup in which cell differentiation, formation of the basal lamina, invasion and proliferation can be monitored and analyzed in an architecturally more native environment (Moreno-Bueno et al., 2009). Figure 8 shows the phenotypic features of EMT in 3D cultures. The most distinct changes are the weakening or disruption of cell-cell junctions and filling of the luminal space. Migratory cells can also invade the adjacent ECM (Kalluri and Weinberg, 2009).

2.5 Cell culture models for studying epithelial cell polarity

2.5.1 Overview of cell culture methods

Cell cultures are either primary cell cultures or cultures of cell lines. In primary cultures, the cells are harvested directly from living tissues. They usually have a limited lifespan determined by their characteristic number of cell cycles. Cell lines, on the other hand, are cells that have been harvested from their original tissues, and the line has been established and maintained in culture. Cell lines have the capacity to divide and proliferate indefinitely. This can be due to either deliberate manipulation or, in the case of malignant cells, due to their inherent ‘immortality’ which in most cases is based on their enhanced telomerase activity (Knuechel and Masters, 1999).

Two-dimensional cell cultures

Cell culture studies are commonly performed on 2-dimensional (2D) surfaces such as microwell plates, tissue culture flasks, and Petri dishes. On such substrata, adherent cells form one-cell layer thick “films”, called monolayers.

2D cell cultures serve as models of cells in tissues. However, they have several limitations and deficiencies that critically detract from their value as valid representations of cell growth and differentiation *in vivo* (Cukierman et al., 2002; Nelson and Bissell, 2006). In primary cultures, dedifferentiation and loss of specialized functions occur when cells are removed from their host tissue and grown on culture plates. Dedifferentiation is believed to be a result of dissociation of the cells from their native environment that harbors supporting and signaling elements required for e.g. the appropriate establishment and maintenance of cell polarity (Guillouzo and Guguen-Guillouzo, 2008). Secondly, the monolayered organization is antagonistic to the formation of three-dimensional structures typical of various functional tissues. In most cases, two-dimensional cultures cannot be considered as valid models of cell growth and differentiation *in vivo* (Yamada and Cukierman, 2007).

Figure 9 depicts a typical setup of growing cells in a 2D cell culture.

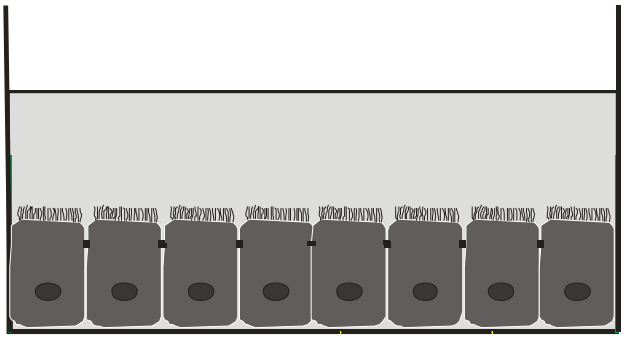


Figure 9 *Cell culture in 2D.*

Due to the deficiencies described above, there is an obvious need to develop tissue culture systems which can either promote redifferentiation of laboratory cell lines or prevent primary cell lines from dedifferentiating (Masters, 2000). This has been pursued by two strategies: growing cells on filters or on microcarriers in suspension that promote polarization (Yamada and Cukierman, 2007).

Filters

The use of filters is based on a strategy of compartmentalizing soluble and solid growth signals in order to favor proper polarization and differentiation. This is achieved by taking advantage of the porosity of a filter, which allows directional exposure of the cells to growth-promoting signals vectorially in a manner that mimics the situation in tissues (Grobstein, 1953). In some cases, the filters or growth supports can be engineered to have

a biochemical composition similar to that of a polarization-supporting basement membrane. Filters allow the formation of a vectorial inductional field similar to that occurring *in vivo* (Handler et al., 1984).

The filters can be placed on a cell culture dish, or special well inserts that allow the study of both surfaces of a cell monolayer can be used (Matlin and Simons, 1984). Such an arrangement is especially well suited for studying epithelial cell migration, development and tissue modeling. Epithelial cells differentiate better on filters than on a bare tissue culture dish (Handler et al., 1984; Steele et al., 1986). Filters also allow the study of migration and invasion. In invasion studies, the cells can be plated on one side of the filter and the cells that have migrated through the filter can be counted on the other side (Lu et al., 2001).

Figure 10 shows a setup in which cells are grown on top of a filter-well insert. The filter can also be laid on the cell culture dish.

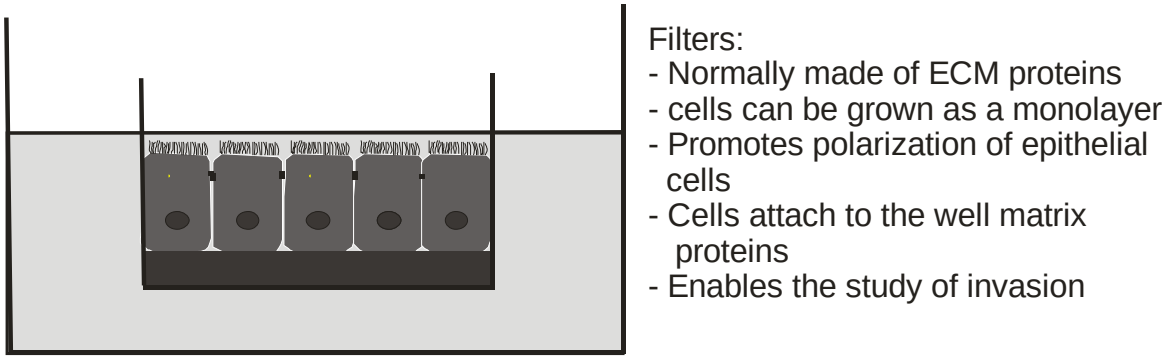


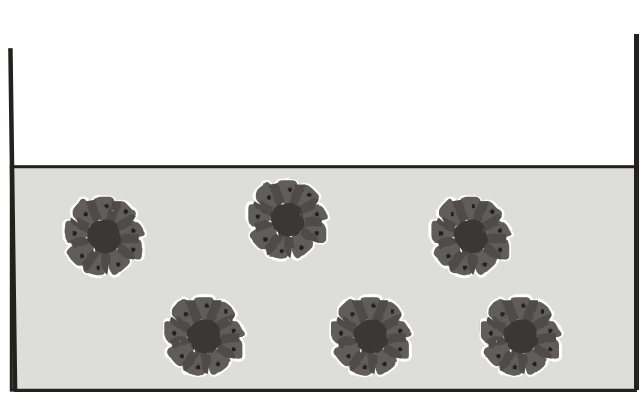
Figure 10 *Cell culture on top of a filter-well insert. Filters can be placed on a cell culture dish or in specific filter well inserts.*

Microcarriers

Microcarriers are small spheres, less than 500 mm in diameter. Their enormous (relative to weight and volume) surface area of up to 500 cm^2/g can support the culture of large numbers of cells in small volumes. The suspension technique was originally designed mainly for bioproduction (Malda et al., 2003). The cells attach to and grow on the surface and crevices of the microbeads which are kept in suspension culture (Malda et al., 2003).

Microcarriers have been successfully used for instance to expand mouse embryonic stem cells 50-fold without inducing differentiation (Abranches et al., 2006; Fernandes et al., 2007; Yang et al., 2007). The beads can be coated, for example, with any number of proteins, such as collagen or laminin. The purpose of the coating is usually to promote cell adhesion to the beads. Some microcarriers are made of a porous material similar to a sponge. Both coated and porous beads are used in suspensions. They are cultured in a bioreactor, in a packed bed device, or in a rocked dish to achieve varying yields and mimick 3D phenotypes (Justice et al., 2009). The same material that is used in porous microbeads can be used as sponges, i.e. the porous material provides cues for the cells to grow (Pampaloni et al., 2007). Figure 11 shows a setup of growing cells on microbeads.

The cells attach and grow on the surface of the beads that are kept floating in the culture medium.



Microcarriers:
- Small beads in suspension
- Promotes polarization of epithelial cells, round surface is thought to mimic 3D structures
- Beads can be coated with various proteins
- Cells attach to the matrix proteins on the beads
- Enables culture of large number of cells in small volumes

Figure 11 *Cell culture with microcarriers. Cells are grown on carrier beads that are kept in maintained suspension culture; the cells attach to the round microcarriers mimicking the 3D structure of organs.*

3D culture models

3D tissue models bridge the gap between traditional 2D cell cultures and tissue culture and animal models (Griffith and Swartz, 2006; Rangarajan et al., 2004). Thus, they fulfill the need to expand from the reductionist 2D approaches to a higher level of cell/tissue hierarchy in order to reveal the underlying molecular determinants of tissue architecture and function. The tools generally used in cell and molecular biology in traditional 2D cell cultures can also be applied to 3D tissue models (Yamada et al., 2007).
Table 2 shows the advantages and disadvantages of 3D cultures as compared with 2D cultures.

Advantages of 3D culture	**Disadvantages**
Cell morphology/differentiation more physiological than 2D culture.	Not all cell types can be cultured on the same matrix.
Allows models to define regulatory pathways, genes, cells, etc.	Human matrix components are not easily available.
Important in cell differentiation at different stages of development.	Response of cells and tissues may be limited to a certain stage.
Provides assays including high throughput for testing reagents, inhibitors, stimulators, proteins, etc., of cell/tissue differentiation and cell invasion.	Optimal concentrations and conditions need to be defined.
Can replace feeder layers for stem cell propagation	*In vivo* matrices are not available for all cells.
Used to identify stem cell types.	
Differentiated structures be put back into animals.	
Transparent matrix for easy visualization.	

Table 2 *Advantages and disadvantages of 3D culture (modified from Benton et al., 2009).*

Pieces of tissue (microscopic embryonic organs or slices of intact tissue) can be explanted and cultured *in vitro*. They often retain their original 3D architecture in culture (Gahwiler et al., 1997; Sakai et al., 2003). In contrast to cell cultures, the proper name for them is explants. Tissue explants must be thin (less than 0.3 mm thick) enough to permit adequate oxygenation and nutrition in the tissue interior. Such cultures have been used in tumor biology, e.g. to mimic the conditions of internal nutrient insufficiency associated with tumor necrosis (Hicks et al., 2006).

In 3D models that utilize isolated cells, cell lines, dissociated tissues or stem cells, the cells can be implanted in a 3D matrix scaffold as single cells or as tissue-like aggregates. The 3D scaffolds are usually built up by using purified native molecules such as collagen I and synthetic biomaterials. They provide mechanical support for the cells to bind to. Native extracellular matrices can also be used as scaffolds – they are called reconstituted matrices. In some systems, the cells themselves serve as a source of the extracellular matrix in which they are embedded (Cukierman et al., 2001).

Figure 12 is a schematic drawing of a setup in which epithelial cells are grown inside ECM gel. The cells form spherical structures with a central lumen inside the cyst.

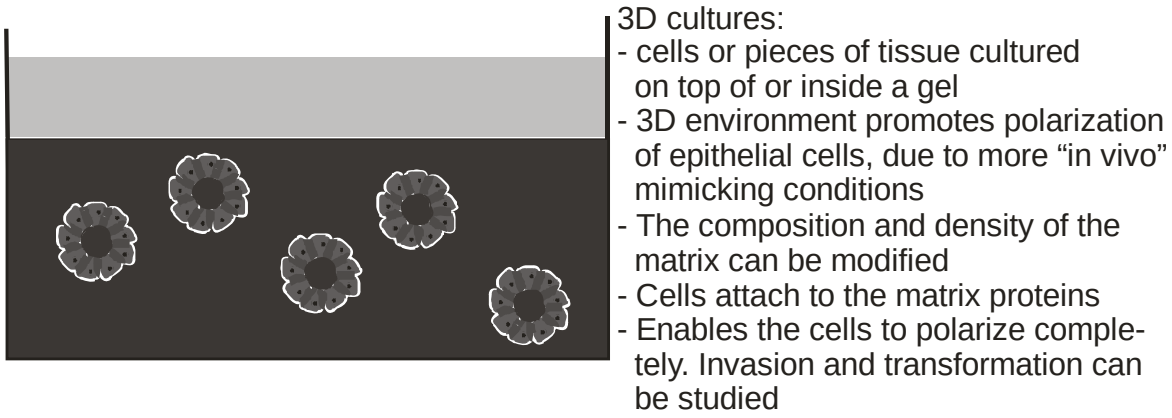


Figure 12 *3D cell cultures. In this figure the cells are grown inside the matrix.*

3D allows culturing more than one type of isolated cell or a tissue fragment in combination with another cell type. For instance, an example of this is the 3D tissue model of human skin, which combines keratinocytes and fibroblasts with cancer cells to simulate human melanoma (Smalley et al., 2006).

Tissue culture conditions

In 3D cultures, oxygenation, nutrition and waste removal depend on simple diffusion. Under such conditions, transport of the molecules becomes a limiting factor as the thickness increases. Restraints in nutrient availability and proper oxygenation in 3D cultures may sometimes mimic the *in vivo* situation and an actual tumor microenvironment better than 2D cultures. In 3D cultures, cells at different depths from

the surface can be considered to represent tumor cells with different nutritional states (Keith and Simon, 2007; Levenberg, 2005).

The composition and stiffness of the extracellular matrix surrounding the cells have a major impact on cell signaling and their behavior. A softer matrix generally promotes more epithelial growth, whereas a denser matrix leads to a more mesenchymal or malignant behavior of the cells (Cukierman et al., 2002; Discher et al., 2005; Grinnell, 2003; Paszek et al., 2005).

The protein composition and the concentration of the matrix proteins determine the density of the matrix. For instance, matrices made of collagen can be engineered to gels that mimic either loose or dense connective tissue, depending on their collagen concentration (Grinnell, 2003). A reconstituted gel of an extract that contains basement membrane components and growth factors, termed Matrigel (or EHS matrix or Cultrex), is especially suitable for the growth and differentiation of epithelial cells (Kleinman and Martin, 2005).

Different types of matrices can also have experimental drawbacks. Not all cells thrive on the same matrix (Cukierman et al., 2002; Discher et al., 2005). To ensure the best outcome, it is crucial to select an appropriately matched 3D *in vitro* matrix for the study (Yamada and Cukierman, 2007).

Cell and tissue polarity

The development of 3D culture systems has especially benefited studies aimed at elucidating the genetic and non-genetic determinants of epithelial cell polarity in normal and cancer cells. 3D culture systems have also been widely used in studies on genes involved in cell adhesion and cell polarity in the pathogenesis of cancer (Nelson and Bissell, 2006).

The epithelia in organs are organized either as flat surfaces of various thickness or as lobular or tubular structures. In both cases, they lie on or are surrounded by a basement membrane. In glandular tissues, epithelial cells are organized into spherical 3D structures surrounded by a basement membrane with a central lumen. The benefits of a 3D matrix culture are obvious especially in glandular and tubular structures, since the overall organization of glandular tissues is lost on a 2D tissue culture substratum. In some cases, the native organization and differentiated functions can be restored or maintained when the cells are transferred from 2D to 3D culture conditions (Griffith and Swartz, 2006; Nelson and Bissell, 2006).

3D cultures provide more appropriate conditions for investigating the mechanism formation and maintenance of glandular lumen. They are also well suited for studying the genetic determinants of cell polarity, as well as the role of cell-cell and cell-matrix contacts. The use of well characterized primary cells or cell lines in 3D also allows exploration of intracellular signaling events that are activated in response to microenvironmental stimuli (Nelson and Bissell, 2006).

Composition of the 3D matrix

The most widely used matrices in 3D culture systems are made of ECM molecules. The most common 3D matrices are either gels or sponges. Sponges are ready-made lyophilized gels generally composed of matrix materials, typically collagens, and engineered to have a desired pore size that allows cell attachment and growth. Cells are placed on top of the sponge and the porous material gives cues for cell polarization. On the other hand, gels enable the mixing of the cells to the matrix, when the gel is in liquid form. The cells can also be seeded on top of the gel. The most common proteins used in gels are collagen in its native or denatured form (gelatin) and laminin (Yamada and Cukierman, 2007).

In a typical 3D culture setting, dissociated cells are embedded in the matrix by mixing the cells into liquid ECM gel. In some 3D techniques, the cells are planted on top of a solid matrix, and the ECM proteins are then added to the cell culture medium to provide proper matrix signaling also on the side of the growing cells that is not surrounded by the underlying matrix (Debnath et al., 2003).

Matrigel™ is a reconstituted basement membrane eluted from the Engelbreth–Holm–Swarm (EHS) tumor grown in mice; it is suited for culturing epithelial cells. It is rich in basement membrane components, type IV collagen, laminin and heparan sulfate (Kleinman et al., 1982, 1986).

Extracel™ is a product composed of hyaluronan, gelatin and the cross-linker polyethylene glycol diacrylate (PEGDA). Hyaluronan makes it a compressible hydrogel with a structure similar to that of cartilage. The composition of the hyaluronan-based gel can be varied by adding other ECM components, and the stiffness of the gel can be modified by adjusting the cross-linking conditions (Shu et al., 2006).

AlgiMatrix™ is a non-animal-based ready-to-use sponge made of lyophilized alginate gel (Shapiro and Cohen, 1997). Alginate is a polymeric sugar obtained from brown seaweed that gelifies in the presence of divalent cations forming a negatively charged hydrogel similar to glycosaminoglycans. AlgiMatrix™ allows cells to invade the porous gel and to secrete endogenous ECM components supporting an *in vivo*-like morphology, structure and behavior. These microenvironments are especially suited for the growth of primary and stem cells in spheroid culture (Kim, 2005; Kunz-Schughart et al., 2004).

Stiffness of the matrix

Elastic modulus is the measure of the stiffness/compliance of the extracellular matrix. For instance, in collagen-based matrix it depends on collagen content, the thickness of a single fiber, and the extent of interfibrillar cross-links. The cross-links define the stability and deformability of the tissue scaffold (Shoulders and Raines, 2009).

The compliance of the extracellular matrix has a role in regulating numerous cellular functions both *in vivo* and *in vitro* (Discher et al., 2005; Paszek et al., 2005; Pelham and Wang, 1997). Many cells alter their growth and differentiation status according to the mechanical stiffness of the ECM *in vitro* (Discher et al., 2005).

Cells sense matrix rigidity via integrin-mediated adhesion and their downstream mechanosensor protein signaling machineries (e.g. via talin and p130CAS) (Giannone and Sheetz, 2006). The integrin receptors on the cell surface and the contractile cytoskeleton pull against the extracellular matrix to sense the stiffness of their microenvironment (Ghosh et al., 2007; Ingber, 2006; Vogel and Sheetz, 2006). Increased substratum stiffness reinforces cellular protrusions and cell motility (Peyton et al., 2008; Ulrich et al., 2009). A rigid matrix stimulates directed cell migration, similar to chemotaxis. The cells migrate towards the substratum of greater stiffness; this process is called durotaxis (Lo et al., 2000; Li et al., 2005; Isenberg et al., 2009). In contrast, a soft matrix does not reinforce the formation of focal adhesions and cytoskeletal contractility, but rather promotes cell rounding (Ulrich et al., 2009).

In tissues, the stiffness of the matrix can vary in different microenvironments: loose versus dense connective tissue, soft versus hard tissues (such as bones and teeth), and early versus late stages of wound healing. The matrix stiffness affects the integrin receptor distribution on the cell surface, the types of cell adhesions established and, consequently, the cytoskeletal structures formed (Cukierman et al., 2001; Katz et al., 2000; Walpita and Hay, 2002).

Matrices with different degrees of stiffness have different effects on the intracellular signaling via Rho kinase and Rac which, for the most part, mediate integrin-cytoskeleton signaling (Pankov et al., 2005; Paszek et al., 2005; Wozniak et al., 2003). Generally, a higher degree of stiffness enhances cell proliferation, and in some cases can promote neoplasia (Paszek et al., 2005; Pelham and Wang, 1997; Mammoto and Ingberg, 2009).

Different 3D models provide a wide range of matrix stiffness, mimicking different tissues in living organisms (Discher et al., 2005; Engler et al., 2006; Paszek et al., 2005). Table 3 shows the compliance of different tissues and materials.

Compliance of biological tissues and culture models

	Compliance (shear modulus, Pa)
Mammary adipose tissue (human)	300-1000
Kidney (rat)	400-600
Brain (rat)	200-400
Collagen gel	10-100
Polyethene glycol gel	20-400
Polyacrylamide gel	100-10000
Polystyrene	10^{12}
Glass	10^{12}

Table 3 *The compliance/ stiffness of some biological tissues and cell culture environments.*

Tensengrity is a concept used originally in mechanics and later applied to cell biology. It defines a structural principle based on the interacting components that are in constant compression within a network of separate structural components of continuous tension. Ingberg (2006) applied these mechanical principles to biology and created the theory of tensengrity. According to the theory, cells can behave as structures in which their shape results from balancing tensile and hydrostatic forces (Ingberg, 2006). The actin

cytoskeleton binds to ECM via integrins, and it stretches the cell so that it forms a tent-like structure (Glogauer et al., 1997; Chicurel et al., 1998; Chen et al., 1999; Meyer et al., 2000; Wozniak et al., 2000). Thus, there are more stress fibers and more tensed-up cell phenotypes in a denser matrix. On the other hand, in a soft matrix the cells have a more rounded phenotype (Ingberg 2006).

Stiffness of matrix and cancer

Cells are in intimate contact with the stromal (ECM) components of tissues. The stiffness or rigidity of ECM enhances cell growth and survival and promotes migration (Lo et al., 2000). A denser matrix disrupts tissue morphogenesis by increasing cell tension (Paszek et al., 2005). It has only recently been clarified that physical environmental factors, such as stiffness of the cellular substrates, control many aspects of cellular behavior (Ingberg 2006). The stiffness of tissues has been exploited in clinical medicine to detect cancer (Buthcher et al., 2009; Sinkus et al., 2000).

Collagen is the most abundant structural macromolecule in the stroma. It contributes significantly to the tensile strength of tissue, so that a higher collagen content correlates with increased stiffness of the ECM (Kolacna et al., 2007).

The metabolism of collagen is deregulated in cancer. Progression of cancer is accompanied by elevated expression and deposition of collagen. Also its organization may be altered (Jodele et al., 2006).

Matrix metalloproteinases (MMP) are critically involved in the molding of the matrix and, thus, they affect the density of the cellular microenvironment. An elevated level of collagen degradation due to MMPs enhances tumor progression (Jodele et al., 2006). MMP-mediated collagen remodeling creates a space for the cells to migrate, produces substrate cleavage fragments with biological activity, modifies cell adhesion to regulate tissue architecture, and activates, deactivates, or alters the activity of several signaling molecules (Page-McCaw et al., 2007). As a rule, high levels of MMPs correlate with a poor prognosis in cancer patients (Tetu et al., 2006).

Cell migration

A migratory phenotype of cells is required for the homeostasis of tissues throughout the entire lifespan of an individual. It is especially important during the development of embryonal tissues. During gastrulation, the cells divide and migrate to their correct embryonic layers. Later on, tissue renewal is dependent on the migratory phenotype of the precursor cells and their descendants. For instance, in wound healing, the polarized epithelium undergoes epithelial-mesenchymal transition with cells covering the wound and displaying the newly acquired migratory phenotype. Cell migration takes place also in pathological processes such as cancer. Cells from the primary tumor can migrate and invade the adjacent tissues (Horwitz and Parsons 1999).

There are differences in morphology, proliferation, and directionality of migration when cells are cultured in 3D, as compared with cells grown in 2D on the same matrix (Cukierman et al., 2001; Pankov et al., 2005; Zaman et al., 2006). The differences between the 2D and 3D culture models are discussed later.

Comparison of 2D and 3D cultures

The major advantages of 2D cultures are the convenience of use and higher cell viability. In biological terms, however, 2D cultures are far from ideal. They cannot critically elucidate phenomena that depend on signals from the extracellular matrix, nor events in which the intercellular – and particularly directional – communication play a role in maintaining tissue architecture. Briefly, most 2D culture systems completely lack a proper extracellular matrix with its fibrous skeleton and the growth factors embedded in it. They are also devoid of a spatially organized cell-cell network which *in vivo* provides numerous growth and survival signals and guides cell differentiation. In addition, the rather restricted assortment of growth substrata routinely used in 2D cultures does not allow simulation of the myriad distinct environmental conditions permitting characteristic behavior of individual cell types and their differentiation in specific niches. Most importantly, the rigid and physiologically deficient 2D growth conditions may force adaptations that do not represent either geno- or phenotypic features of the same cells *in vivo* (Lee et al., 2008).

Numerous studies have shown that cells grown on flat 2D tissue culture substrates differ in their morphology, cell-cell and cell-matrix interactions, and differentiation from those grown in more physiological 3D environments (Birgersdotter et al., 2005; Cukierman et al., 2002; Griffith and Swartz, 2006; Nelson and Bissell, 2006). Discrepancies occur, for instance, in studies focusing on the effects of gene ablation on cellular phenotype and in studies elucidating the functions of specific proteins (Knight and Shokat, 2007). Apart from the changes in the specifics of protein expression, several studies have shown that forcing the cells into 2D culture also brings about more global derangements, such as alterations in cellular metabolism (Chang, 2004; Abbot 2003; Cukierman et al., 2001).

2.5.2 Madin-Darby canine kidney cell culture models

MDCK II

The Madin-Darby canine kidney (MDCK) cell line is derived from the distal tubules of canine kidney (Leighton et al., 1969). It is a well-characterized model used widely to study epithelial polarity (Rodriguez-Boulan, 1983). MDCK cells form a polarized epithelial monolayer when grown in 2D. The cell line tends to differentiate spontaneously, and it forms highly polarized monolayers in culture. Functionally, it displays the characteristics of a distal kidney tubule (Kreisberg and Wilson, 1998). The polarity can be

enhanced by growing the cells on a support resembling their native environment (Gospodarowicz et al., 1983; Simons and Fuller, 1983).

Polarized MDCK cells have a distinct domain structure. They have a clearly delineated apical cell surface facing the culture medium. The apical face is covered with microvilli. The basolateral cell surface is attached to the substratum, and is laterally in contact with the neighboring cells. The two surface domains are separated by a junctional complex. The functional differences between the two sides of the epithelium are illustrated by their unique protein and lipid compositions (Richardson and Simmons, 1979; Louvard, 1980; van Meer and Simons, 1982).

In their natural habitat, i.e. the kidney, MDCK cells are organized as tubules, but this spatial arrangement cannot be emulated in 2D cultures. On the other hand, a native structure can be achieved when the cells are grown in 3D collagen gel or in Matrigel. In 3D, the cells form polarized structures resembling kidney tubules. The equivalents of the tubules in 3D cultures are cysts in which the apical side of the cell faces towards the lumen of the cyst, and the basal side towards the extracellular matrix. Scatter factor / hepatocyte growth factor promotes tubegenesis in cultures (Brinkmann et al., 1995).

In suspension, without the support of the matrix, MDCK cells organize into a cyst with an inverted polarity. The cells form cell-cell junctions laterally and secrete the basal membrane proteins inside the cyst. Consequently, the polarity of the cells becomes inverse relative to MDCK cells grown in solid matrix. This demonstrates the importance of the matrix; binding of the cells to the vectorially distributed ECM proteins guides the orientation of the polarization (Wang et al., 1990).

Ts v-src MDCK cells

Disassembly of junctional complexes and loss of cell polarity are among the hallmarks of cancer (Hanahan and Weinberg, 2000). Earlier studies with MDCK and other epithelial cells have demonstrated the importance of junctional complexes in the establishment of cell polarity. The cadherins of adherens junctions guide the tight junctional protein ZO-1 to the apical half of lateral walls. Subsequently, cadherins orient themselves to form adherens junctions and help to maintain the apico-basal axis of the polarized epithelium (Rodriguez-Boulan, 1983). It was later shown that many of the molecular components and cell junctions are targets of tyrosine kinase signaling proteins (Tsukita et al., 1991).

Walter Birchmeier and Jurgen Behrens and their collaborators (1993) developed a MDCK epithelial cell line that stably expresses a temperature-dependent mutant of v-src protein (Behrens et al., 1993). For that purpose, MDCK I cells were transformed with a temperature-sensitive mutant of ts 31 v-Src. The cells exhibit an epithelial phenotype at the non-permissive temperature, i.e. at the temperature at which the kinase activity of v-Src is suppressed (40.5° C). On the other hand, they acquire a transformed, fibroblast-like morphology when they are cultured at the permissive temperature (35 °C) at which the kinase activity of the src protein is manifest (Behrens et al, 1993).

Comparison of the biochemical and functional properties at non-permissive and permissive temperatures allows detailed examination of the changes associated with the

loss and gain of a polarized phenotype and other correlates of malignant transformation (Behrens et al., 1993; Solberg et al., 1992). Several studies clearly show that activation of v-Src weakens cell-cell adhesion by disrupting the adherens junctions and by interfering with the cell-matrix adhesion at focal adhesion sites (Behrens et al., 1993; Takeda et al., 1995). V-src interferes with cell adhesion in cultured cells, and its low expression has been associated with disturbance of the junctional complex, particularly in the adherens junctions (Rohrschneider, 1980; Tsukita et al., 1991; Hirst et al., 1986; Pasquale et al., 1986; Warren and Nelson, 1987).

2.5.3 Other cell lines commonly used to study epithelial cell polarity and transformation

Breast cancer cell lines MCF-10A, Ras-MCF10 A and MCF 7

MCF-10A cells are an immortalized, non-transformed epithelial cell line derived from human fibrocystic mammary tissue (Soule et al., 1990; Tait et al., 1990). MCF-10A cells are an excellent model for studying epithelial cell biology. When plated in a mixture of collagen and laminin, they form 3D structures that resemble acini of the human breast (Debnath et al., 2003).

The Ras-transformed MCF10A cells appear more mesenchymal (fibroblastic) in their morphology, in the organization of their cell-cell junctions, and in their actin cytoskeletons (Kinch et al., 1995). Normal MCF10A cells grow in culture as tightly clustered epithelial colonies. The Ras-transformed MCF10A cells are more loosely arranged and contain large bundles of actin filaments (stress fibers) that extend across the cells and terminate in focal adhesions (Kinch et al., 1995; 1996).

MCF-7 is a breast cancer cell line that retains many characteristics of differentiated mammary epithelium, such as the ability to form a tight epithelial monolayer (Cowley 2006). It also expresses estrogen receptors and has the capacity to form dome-like structures indicative of transepithelial fluid movement (Soule et al., 1973). MCF-7 cells are widely used as a model to study breast cancer progression (Dupont and Le Roith, 2001; Farooqui et al., 2006).

Colon cancer cell lines: Caco-2, HT-29 G+, HT-29 rev

Caco-2 is a colon adenocarcinoma cell line (Fogh et al., 1977). Caco-2 cells differentiate spontaneously and are used as a model of intestinal carcinogenesis (Rousset, 1986; Jumarie et al., 1991).

HT-29 is a colon carcinoma cell line. Its state of differentiation depends on the culture conditions. Under standard conditions with glucose, HT-29 cells remain undifferentiated (HT- 29 G+). On the other hand, when cultured in the total absence of sugar (HT-29 G-) they polarize (Pinto et al., 1982).

The cell lines mentioned above are some of the most widely used both in 2D and in 3D cell culture studies. In addition to these, several other cell lines are used in organ-specific studies of carcinogenesis in 3D.

Some of the commonly used cell lines for studying cancer in 3D cultures are listed in Table 4.

Cancer	Cell line	Cancer	Cell line	Cancer	Cell line
Liver	Hep3B HepG2 HLE HLF	Colon	COLO201 COLO205 DLD-1 HT-29 HTC116 SW480 Sw620	Mammary	BT20 BT474 Bt549 HCC1954 MCF7 MCF10 MDA-MB-231 SK-BR-3 T47-D ZR-75-30
Pancreas	PxPC-3 Canpan-1 Canpan-2 MiaPaCa-2 Panc1	Prostate	Du145 PC-3		
Lung	A549 Calu-1 NCI-H23 NCI-H226 NCI-H460 NCI-H1650	Skin	MDA-MB-135S		
		Ovary	SK-OV-3 OVCAR-3		

Table 4 *Commonly used 3D cell models for cancer*.

3 Aims of the study

Epithelial tissues maintain their polarized structure and functional properties during the life span of the tissue and under conditions of continuous tissue renewal. The balance between proliferation, polarization, apoptosis and proper differentiation is crucial for the homeostasis of tissues.

Much of the knowledge on the determinants of the normality of epithelial cells and their derangements in cancer is derived from 2D cell culture studies. In 2D, the cells have adapted to grow as a monolayer on a flat surface. However, it is obvious by inference, and it has also been demonstrated experimentally, that 2D culture models are a poor and often misleading representation of the actual conditions confronted by the cells and of their supracellular architecture in living tissues. More biologically relevant model systems are therefore required. Attempts have been made to develop 3D culture systems that could serve as more realistic models of cell growth and differentiation of cells *in vivo*.

MDCK cells stably transfected with a gene encoding a temperature sensitive v-Src oncogene product is a well-characterized cell line. It is especially well suited for studies on the mechanisms of establishing and maintaining an epithelial phenotype in normal tissues and its changes in malignant transformation. Earlier studies have shown that ts-src MDCK cells also serve as an excellent model for studying epithelial differentiation in 3D.

The aims of the present study were:

1. To design and optimize novel methods to culture, immunolabel and image MDCK and ts-src MDCK cells in 3D culture conditions, and to develop methods for monitoring the state of adherens junctions, cell polarity and mitochondrial function in living cells.

2. To determine the effect of ECM composition and stiffness on the polarization and transformation of ts v-src MDCK cells in 3D cell cultures.

3. To analyze the effect of v-src transformation on the phenotype and the gene expression of ts-src MDCK cells in 2D and in 3D cell cultures.

4. To study the role of survivin and its regulator PTEN in cell polarization and transformation, and in the determination of cellular phenotype and cell fate in src-transformed MDCK cells.

4. Materials and Methods

4.1 Cell lines, reagents and antibodies

The cell lines, antibodies and some other non-routinely used reagents are listed in Table 5. The references and the original publication in which the cell line/antibody/reagent was used are indicated by their Roman numerals.

Table 5*. The list of cell-lines, reagents and antibodies used in this work.*

TABLE 5

Cells/Reagents/Antibodies	Source	Original publication
Cells		
MDCK II	ATCC, USA	I-III
Ts-src MDCK	Behrens et al., (1993), Berlin, Germany	I-III
E-Cadherin-GFP ts-src MDCK	Töyli et al., (III), Oulu, Finland	III
Reagents for cell studies		
TGFβ and anti-TGFβ	R&D Systems, USA	I
PP2 (4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine)	Invitrogen, USA	II-III
Integrinα2β1-blocking antibody, MDCK medium	Matlin et al., (2005), Heidelberg, Germany	III
Antibodies/Actin indicators		
E-cadherin mouse mAb (610181)	Transduction Laboratories, USA	II
E-cadherin mouse mAb (rr1)	Developmental Studies Hybridoma bank, USA	I-III
Pan-cadherin rabbit pAb (71-7100)	Invitrogen, USA	II
β- catenin mouse mAb (C19220)	Transduction laboratories, USA	I-III
ZO-1 rat mAb	Chemicon, USA	I-II
Survivin mAb	Abcam, UK	II-III
Src py mAb	Cell Signaling Technology, USA	II-III
Src phosphorylated mAb	Cell Signaling Technology, USA	II-III
V-Src (avian) mAb	Cell Signaling Technology, USA	II-III
PTEN mAb	Upstate, USA	II-III
Cleaved caspase mAb	Cell Signaling Technology, USA	II-III
Texas red antimouse mAb	Molecular Probes, USA	I
Alexa 488 phalloidin	Invitrogen, USA	II-III
Alexa 568 phalloidin	Invitrogen, USA	II-III
Rhodamine phalloidin	Invitrogen, USA	II-III
TRITC anti-rat antibody	Invitrogen, USA	I-III
DAPI (4',6-diamidino-2-phenylindole, dihydrochloride)	Invitrogen, USA	II-III
Hoechst33342	Invitrogen, USA	II
Mitotracker Green FM	Invitrogen, USA	II-III
Mitotracker Orange CM-H2TMRos	Invitrogen, USA	II-III
Collagenase A	Boehringer Mannheim GmbH, Germany	I
Hyaluronidase	Sigma, USA	I

4.2 Cell culture (I-III)

4.2.1 Two-dimensional cell culture (I-III)

MDCK II cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's medium with Earle's salts (E-MEM), supplemented with 2 mM glutamine, 10% FCS, antibiotics and antimycotics (I-III).

The ts-src MDCK cells were provided by Prof. Walter Birchmeier and Dr. Jürgen Behrens (Max Delbrück Center for Molecular Medicine, Berlin, Germany), (Behrens et al., 1993). Ts-src MDCK cell line stably transfected with EGFP-tagged pN1-ECad (see below) was cloned and established in our laboratory (III). Both cell lines were grown in in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 2 mM glutamine, 10% FCS and antibiotics/antimycotics at 35 ^{o}C and 40.5 ^{o}C.

4.2.2 Cell suspension cultures (III)

MDCK and ts v-src-MDCK cells were incubated with a $\alpha 2\beta 1$ integrin-blocking antibody (a kind gift from Dr Kai Matlin, Heidelberg, Germany) at a dilution of 1:10 in flexiPERM reusable cell culture chambers on Petri dishes (Greiner Bio-One) with D-MEM and 10 % FCS.

4.2.3 Construction of pN1-ECad-EGFP and transfection of ts-src-MDCK cells (III)

The mouse E-cadherin cDNA (NCBI-gi:68533543:1-3314) in pBluescript plasmid was cloned by PCR and inserted between the BglII and KpnI restriction sites of the pEGFP-N1-linker expression vector using the In fusion^{TM} cloning system (Clontech). This yields to a fusion of EGFP, via a short linker sequence, to the C-terminus of E-cadherin, henceforth designated pN1-ECad-EGFP. The linker sequence had been previously cloned to pN1-EGFP (Clontech) between the KpnI and AgeI restriction sites.

In order to obtain a stable expression of the E-cadherin-EGFP fusion protein in ts-src-MDCK cells, the pN1-ECad-EGFP construct was transfected to the recipient cells by electroporation using the NucleofectorTM II equipment (Amaxa Biosystems) with the IngenioTM electroporation kit and solutions (Mirus). Neomycin-resistant transfectants, henceforth designated as cadherin-GFP ts-src MDCK cells, were selected over a period of 10 days with the G-418 antibiotic in 400 μM concentration (Roche). Clone no 1 was picked from three similarly behaving clones for the further studies.

4.2.5 Three-dimensional cell culture (I-III)

In order to investigate the role of different ECM protein on the polarization, the MDCK and ts-src-MDCK cells were seeded and grown in different ECM environments. Matrices containing various amounts of ECM proteins, mixtures of collagen I (stiff matrix), a mixture of ECM proteins (collagen I/laminin /proteoglycans /collagen IV) and Matrigel (loose matrix) were prepared as described in I. In order to establish the 3D-cultures, the cells were seeded inside the gel. The culture medium was D-MEM supplemented with 2 mM glutamine, 10% FCS (for the culture in collagen I or in collagen-laminin mixture) or 10% horse serum (for the culture in Matrigel) and antibiotics/antimycotics (I).

For the publication II and III, the cells were cultured in Matrigel or in collagen-Matrigel mixtures as described in II. For immunofluorescent microscopy the cells were planted on top of an ECM gel and fed with D-MEM including 2% ECM (Matrigel and/or Collagen I) proteins in the cell culture medium, as described by Debnath and Brugge (2005) (II-III).

4.3 Microscopy (I-III)

4.3.1 Processing the cells in 2D for confocal microscopy (I-II)

Cells grown in 2D were fixed using optimized procedures for each antibody and cell line. Detailed procedures are described in the original publications (I-II).

4.3.2 Processing the cells in 3D for confocal microscopy (I-III)

In order to investigate the role of ECM on the polarization of MDCK and of ts-src MDCK cells (I), the cells were grown inside 3D gels. The cultures were then treated enzymatically by using 0.05% collagenase A (for collagen I and for the mixture of collagen and basal membrane proteins), or by using a cocktail of collagenase A and 0.56% Hyaluronidase (for Matrigel). Thereafter the cells were fixed with 4% formaldehyde/0.2% Triton X-100 in PEM-buffer. After additional 0.1% Triton X-100 treatment, the gels were incubated with the primary antibody overnight and then with the secondary antibody for two hours at $+4^{o}C$ as described in I.

For the publications II and III, the method described above was further modified by omitting the peptidase treatment. This was done to avoid any potentially distorting effect on the 3D-architecture The cells grown in 3D mixture of Matrigel and collagen I were fixed with 2% formaldehyde in phosphate buffered saline (PBS) for 20 min, blocked with 0.1 % Bovine serum albumine (BSA), 0.2% Triton X-100, 0.05% Tween-20 and 10 % Fetal calf serum (FCS) in PBS for 30 min, incubated overnight at $+4\ ^{o}C$ with the primary antibody, followed by for one hour at room temperature with the secondary antibody and DAPI. The samples were refixed with 2 % formaldehyde in PBS for 20 min at room

temperature, washed and mounted with Immu-Mount (II-III). Antibodies to E-cadherin, β-catenin, actin, ZO-1 and to cleaved caspase were used to visualize the respective proteins, used as markers for polarity and apoptosis.

4.3.3 Confocal imaging (I-III)

For the publication I, the 2D and 3D samples were stained as described for immunofluorescence microscopy and viewed under Zeiss 510 confocal laser scanning microscope, equipped with an Argon laser (488 nm) and HeNe laser (543 nm). Scanning was performed in XY-plane at 0.3μm intervals by using 63x oil objective, a 505-530-nm band pass filter for green emission and a 560-nm or a 630-nm low pass filter for red emission. The Z-sections were created later with the computer software (I).

For the publications II and III, the fixed specimens were viewed under Olympus FluoView1000 confocal microscope using 63x oil objective and appropriate filter sets for blue, green and red channels. For 2D specimens, from 6 to 8 optical layers, and for 3D cell cysts, from 20 to 30 layers were collected using a step size 0.5 μm through the specimen. The images were collected and saved using the Olympus software (II-III).

Co-localization analysis of E-cadherin with β-catenin was carried out using the Olympus FluoView1000 software (II).

4.3.4 Transmission electron microscopy of 3D cells (I)

MDCK and ts-src-MDCK cells, grown in 3D gels, were fixed in 4% paraformaldehyde, and further processed for transmission electron microscopy as described in I.

4.3.5 Live-cell microscopy of pN1-ECad-EGFP v-Src MDCK cells (III)

The ts pN1-ECad-EGFP v-Src MDCK cells were mixed in ice-cold Matrigel in a 6-well cell culture dish (BD). Cell culture medium (D-MEM with 10% FCS) was added on top of Matrigel after polymerization of the gel. The cells were grown in an Okolab cell culture chamber (Okolab, Italy). The use of Olympus Cell P video microscope (Olympus) was used for the real time monitoring of the cells.

4.3.6 Measurement of mitochondrial activity (II-III)

For the measurement of mitochondrial activity at non-permissive and permissive temperatures, ts-Src- MDCK cells were grown in suspensions for 12, 15 and 20 hrs or in 3D Matrigel for 1 to 8 days. They were then labeled with a mixture of Mitotracker Green (0.2 μM, Invitrogen, USA) and Mitotracker Orange CM-H_2TM Ros (0.2 μM, Invitrogen USA) for 15 min in the cell culture incubator. For the measurement of mitochondrial

activity, the cell cultures were illuminated with an Olympus Cell M video microscope using a 40 x water immersion objective and appropriate filters for green and red channels. The details of the measurement are described below. After each measurement, the cell culture dishes were returned to the cell culture incubator and experiments repeated every 24 hrs for 8 days.

In order to quantitate the mitochondrial activity of the cultures labeled as described above, the following analysis was performed. The image intensities of selected regions of interest (ROIs) in the samples were measured using the Olympus CellM software. The ratio of the intensity on red channel to that on green channel was taken as a measure of the proportion of active mitochondria as compared with the total mitochondrial mass. This ratio, in turn, was taken as a measure of the metabolic rate. Thus, the lower the ratio, which henceforth is called metabolic index, the lower the metabolic rate of the cells in ROI. The statistical analysis of the results was performed using Microsoft Excel software.

4.3.7 Cadherin recycling (II)

For the analysis of internalization and recycling of cadherin, the ts-src MDCK cells were cultured in 2D and incubated for 30 min on ice with mouse monoclonal anti-E-cadherin antibody (rr1, recognizing the cytoplasmic tail of E-cadherin) (20 μg/ml). Thereafter the cells were warmed to +40.5 ^{o}C or +35 ^{o}C for a given period of time. The cells were then immunolabeled as described for 2D confocal microscopy. Quantitative analysis was carried out by using the Olympus Fluoview1000 software provided by Olympus after thresholding to eliminate noise and background from the images. The use of co-localization coefficients are the most biologically useful methods for qualifying colocalization. It represents the proportion of pixels from each channel that contribute to the co-localized area e.g. the colocalized pixels as a proportion of all pixel of the channel (Pawley, 2006).

4.3.8 The role of TGFβ1 and anti- TGFβ on lumen formation (I)

In order to study the role of TGFβ1 in cyst formation of ts-src MDCK cells at permissive temperature in growth factor-depleted Matrigel, TGFβ1 was added into the cell culture medium at various concentrations (6.5, 13.3 or 20 ng/ml diluted from a stock concentration of 1 mg/ml, produced in 4 mM HCl containing 1 mg/ml BSA). The cells were cultured in the medium supplemented with TGFβ1 for 24–48 hours, and then immunolabeled for E-cadherin and β-catenin.

In order to block the effects of TGFβ, and prevent lumen formation, anti-TGFβ1 antibody was added to the cell culture medium at a concentration of 1 mg/ml (stock 1 mg/ml in PBS). The cells were cultured for four days with 1 mg/ml anti-TGFβ1 in the replacement medium added every day. The cell cultures were then immunolabeled for E-cadherin and β-catenin.

4.3.9 Inhibition of src-kinase with pp2 (II and III)

In order to inhibit the activity of src- kinase, a Src kinase inhibitor pp2 (Tocris Bioscience, Bristol, UK) was used. It was dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 10mM and used for experiments at a concentration of 50 μM. Pp2 was added to the cell culture medium after trypsination of the cells in 2D, 3D and suspension cultures.

4.3.10 Detection of Apoptotic cells (III)

In order to monitor apoptosis in cell suspensions, the fluorescein 5-isothiocyanate (FITC) Annexin apoptosis kit was used according the manufacturer's instructions (BD Biosciences). MDCK and ts-src MDCK cells at permissive and non-permissive temperatures and with pp2 were cultured in suspensions with integrin α2β1 blocking antibody for 12, 15 and 20 hrs before probing for apoptotic cells. For viewing of apoptotic cells, an Olympus Cell M video microscope using a 40 x water immersion objective and appropriate filters for green and red channel was used. The number of apoptotic cells was counted at each time point.

In 3D cultures, apoptosis was detected in by immunolabeling the cells with an antibody against cleaved caspase 3 as described above. The immunolabeled specimens were scanned with Leica confocal microscope. Apoptotic cells were identified by their positive staining for cleaved caspase 3.

4.4 Gene expression studies (II-III)

4.4.1 Microarray analysis (II-III)

In order to investigate the gene expression in cells grown under different conditions, RNA was isolated from the MDCK and ts-src-MDCK cells grown at permissive and non-permissive temperatures and at permissive temperature with pp2. For the isolation of mRNA, Trizol Reagent Qiagen RNeasy kit was used according to the manufacturer's instructions (Qiagen).

Experimental procedures for GeneChip analysis were as detailed in the Affymetrix GeneChip Expression Analysis Technical Manual. (Affymetrix) We used canine arrays containing approximately 23000 canine transcripts. The expression data was analyzed using Affymetrix GeneChip Operating System (Affymetrix) and dChip software (Li and Wong, 2001).

4.4.2 Real time-PRC (II-III)

In order to validate the observations based on microarray analysis, RNA was extracted from the cell suspensions and from the 2D and 3D specimens according to the instructions of the Qiagen RNAeasy kit (No 74104) and used for the synthesis of cDNA. The expression levels of Rab5, Rab7, Rab8, survivin and PTEN were analyzed using Ubiquitin Specific Peptidase as a control. The forward and reverse primers were designed as described in the original publications (II-III). The Real time-PCR (RT-PCR) analysis was performed using Stratagene Brilliant SybrGreen QPRC Mastermix (600548) and Stratagene Mx3005P RT-PCR equipment. The data was analyzed with MxPro -software (Stratagene, La Jolla, CA, USA).

4.4.3 Western blotting (II-III)

Expression of cadherin, survivin, PTEN and src expression and activity were determined by using total cell lysates that were prepared as follows. The cells from 2D cultures were scraped from dishes, dissolved in cell lysis buffer (50 mM Tris-HCl, pH 7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF, 1 µg/ ml aprotin, 1 µg/ ml leupeptin, and 1 µg/ ml pepstatin) on ice, sonicated and incubated on ice for 30 min. After that the suspension was centrifuged for 2 min x 10 000 g at +4^{o}C. The supernatant was used for the determination of proteins and gel electrophoresis.

The proteins were resolved on 15 % Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane for the immunoblotting. The blots were incubated with the primary antibodies for 2 hours followed by one hour incubation with horseradish peroxidase-conjugated secondary antibody. The blots were developed for 2 min in light with a detection liquid (10 ml 0.1 M Tris-HCl pH 8.5, 35 µl 250 mM luminol in DMSO and 15 µl 90 mM p-coumaric acid in DMSO and 3 µl 30 % H_2O_2) and the emitted light collected using FujiFilm LASS 3000 gel imaging device (FUJI, Japan).

In order to recover the cells from 3D cultures in Matrigel, the specimens were treated by cell recovery solution (BD Biosciences, Franklin Lakes, NJ; USA.) for 30 min on ice the cells were collected by centrifuging at 300 x g for 5 min at +4^{o}C. The pellet was dissolved in the cell lysis buffer and the procedure continued as described above. The expression of PTEN was detected as described above, but a 10% SDS-PAGE gel was used.

The expression and activity of Src was analyzed by Western blotting with the src activity kit including specific antibodies to activated Src (pY416), total cellular Src (36D10) and to avian Src (EC10) according to the instructions given by Cell Signaling Technologies. The matrix was dissolved as described above in 3D samples, the cells were lysed in 1xSDS sample buffer, sonicated, boiled and, after cooling on ice and centrifugation, the supernatant used for protein determination and gel electrophoresis. The

proteins were resolved on 10 % SDS-PAGE and transferred onto nitrocellulose membrane and were immunolabeled as described earlier.

For the analysis of cadherin expression, the cells grown in 2D or 3D environments were separated in Triton soluble and insoluble fractions, the proteins were resolved on 7.5% SDS-PAGE and transferred onto nitrocellulose membrane. After that the blots were incubated with the rr1 (Developmental Studies Hybridoma Bank), anti-E-cadherin (BD Biosciences) or polyclonal rabbit anti-pancadherin for 2 h followed by 1-h incubation with horseradish peroxidase-conjugated secondary antibody.

5 Results

5.1 Development of novel 3D culture models and methods for imaging 3D structures in situ (I-III)

At the time of launching the present project, there were no optimized standard procedures for 3D cultures of epithelial cells. Therefore, setting up relevant experimental systems and optimization of several factors was required. A special attention was also paid to developing techniques for visualization of proteins in living cells by immunofluorescence microscopy. One of the obstacles to overcome was to find ways to guarantee best possible penetration of antibodies through dense matrices and several cell layers typically present in 3D cultures without compromising preservation of the 3D–architecture and cellular integrity.

As part of the methodological developments, novel methods were established to culture ts-src MDCK cells in 3D and visualize relevant marker proteins of cell polarization. This was the topic of publication I. Especially the junctional proteins E-cadherin, β-catenin, actin and ZO-1 were of interest, due to their central role in cellular polarity. In publications II and III, the culture methods were further developed in order to overcome the problem of a deficient penetration of antibodies through the matrix. An aim was to design a technique to avoid the use of peptidases. Their use leads to degradation of the culture matrix. It also breaks up cell-cell junctions and distorts the native 3D structures.

5.1.1 Development and optimization of 3D cultures (I-III)

The improved and optimized 3D-culture techniques are described in publications I, II and III. Three different experimental setups (setup 1 to 3) were tested. In each of them MDCK and ts-src MDCK cells were used and their growth and polarization were monitored by using markers for cell-cell junctions (E-cadherin, β-catenin and ZO-1). The optimization of the immunofluorescent techniques is discussed later in 5.1.2.

The setup 1 is shown in panel A of Figure 13. As discussed in the publication I, the cells were seeded in large volumes of matrix (500 µl) which, when cast, provided a gel of several millimeters in thickness. The matrix material was either Matrigel or collagen I extracted from rat tail tendon. Several ratios of MDCK cells or ts-src MDCK cells per ml of the gel were seeded in matrices of different compositions. Formation of cell-cell junctions and the appearance of a central lumen were evaluated.

In the setup 1, in which the cells were seeded within a large excess of gel, MDCK and ts-src MDCK cells were able to form their typical 3D structures. The large volumes of matrix provided the cells mechanical support. However, the growth of the cells appeared to be uneven so that higher rate of proliferation and higher density of cells was seen closer to the edges of the culture dish when compared with the central area of the culture. This

suggested that in this 3D-culture arrangement, the cells may not have equal access to oxygen and nutrients to guarantee optimal growth throughout the whole gel.

The use of various immunolabeling protocols in setup 1 turned out to be laborious and caused problems. This was because, for the immunostaining procedure, the gel had to be removed from the original cell culture dish and to be cut into smaller pieces prior to permeabilization of the matrix (I).

The setup 2 was designed to solve the problem of the apparent uneven availability and even shortage of oxygen and nutrients in some parts of the thick matrix as seen in setup 1. To overcome the problem, we resorted to a protocol developed by Debnath and Brugge (2003), in which epithelial cells are grown on top of a thin ECM gel with a 2%-ECM in the culture medium. The plan of the setup 2 is shown in panel B of Figure 13. The setup 2 was used in the in the publications II and III for the measurements of mitochondrial activity and for some of the immunostaining experiments in the publication II.

The results by using setup 2 showed that the cells grew well and evenly on top of the rigid gel, suggesting that the protein quantity of the ECM proteins in the cell culture medium is sufficient for proper ECM signaling. The disadvantage of the method was that approximately half of the perimeter of the cellular sphere is devoid of the mechanical support of the surrounding firm matrix. This is a drawback, since recent data suggests that next to appropriate matrix composition, the cells require optimal density of the matrix in order to function and polarize normally (Ingberg, 2006). Immunolabeling techniques were easier with setup 2 in comparison to setup 1. No enzymatic permeabilization of the matrix was needed and for this protocol (II-III).

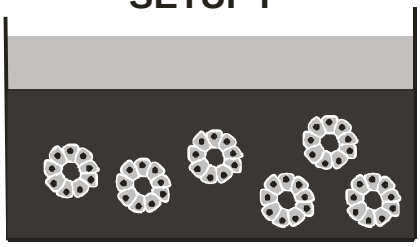
The setup 3 was designed to solve the problem of the lack of mechanical support in setup 2, and at the same time, to enable proper oxygenation and nutrient delivery and easier immunostaining techniques. In the setup 3 the cells were embedded in a small (30 µl) volume of matrix and grown on top of a glass slip in FlexiPERM reusable cell culture chambers as shown in panel C of Figure 13. The FlexiPERM chambers are reusable silicon culture chambers, which can be easily attached to a cell culture dish or a glass slip.

The results showed that setup 3 provided a proper 3D environment with ECM proteins and a sufficient mechanical support. The use of thinner gels enabled easier immunostaining. Most importantly, no protease treatment was needed since a small volume of matrix and use of FlexiPERM chambers on top of the glass slides enabled immunostaining of the cells inside the wells the cells were cultured in. That made transfer of the gel from the original cell culture dish to a separate dish for immunostaining and finally to a cover slip during the immunostaining protocol unnecessary (II-III).

Comparison 3D culture methods used in the study

A

SETUP I



- Cells cultured in large volumes of matrix in a cell culture well

Advantages:

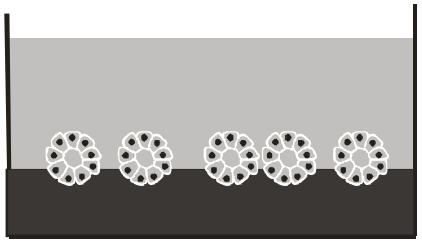
- Enables optical sectioning of the specimen with a confocal microscope
- Matrix contact to all sides of the sphere
- Enables co-cultures and study of invasion

Disadvantages:

- Uneven availability of oxygen and nutrients througout the gel
- Permeabilization of matrix prior to immunostaining, loosing material during staining protocol, thickness of the specimen
- Used in publication I

B

SETUP II



- Cells cultured on top of the matrix with 2% ECM protein dilution in the cell culture medium on top of the gel

Advantages:

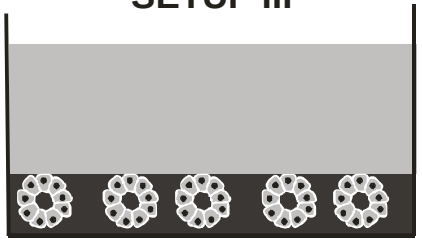
- The immunostaining techniques are easier
- Enables reuse of the specimen for functional testing

Disadvantages:

- The cells are more likely to detatch during the protocol.
- Matrix support only to half of the sphere, may affect the results
- Used in publication II and III

C

SETUP III



- Cells cultured inside a smaller amount of matrix on top of a glass in a FlexiPERM chamber

Advantages:

- The immunostaining techniques are easier, the remains on top of the glass during the protocol. Less material is lost.
- Matrix support around the spheres
- Smaller volume of matrix enable more even proportion of nutrients and oxygen through out the gel
- Enables functional testing
- Used in publication II and III

Figure 13 *Comparison of the 3D cell culture methods used for the study.*

5.1.2 Optimization of immunofluorescence techniques (I-III)

Several factors affect the quality of microscopical specimens and, thus, results of the immunofluorescence microscopic analysis. For the purposes of the present study, special efforts were made to guarantee proper penetration of antibodies and prevention of bleaching and to develop technology to be used in immunofluorescence microscopy of living cells.

3D cell cultures are normally thick and this may cause that the penetration of the antibodies is suboptimal. In order to guarantee a proper penetration of the antibodies without any major interference with the cellular architecture, several modifications of the standard immunofluorescence techniques were tested. One important part of the work was to establish immunolabeling techniques that permit the imaging of 3D spherical structures embedded in the gel.

In order to guarantee proper penetration of antibodies, the gels with cells seeded in them (Setup 1) were taken out from the cell culture well, cut into smaller pieces and treated with a mixture of collagenase I (collagen) and a cocktail of Hyaluronidase A and collagenase (Matrigel) in order to permeabilize the matrix before fixing and immunolabeling the specimen for immunofluorescence microscopy. The MDCK and ts-src MDCK cells were immunolabeled with antibodies against junctional proteins in order to visualize the 3D morphology of the cell cyst. The results are discussed in detail later in 5.2.

The method enabled the visualization of the proteins *in situ*. However one of the major disadvantages of the method was loss of ECM gel and immunostained cells during the manipulation. Excess use of peptidases was also found to dissolve completely the ECM gel and lead to breaking up of cell-cell contacts. Also physical thickness of the specimen caused problems in finding the focus in confocal microscopy (I).

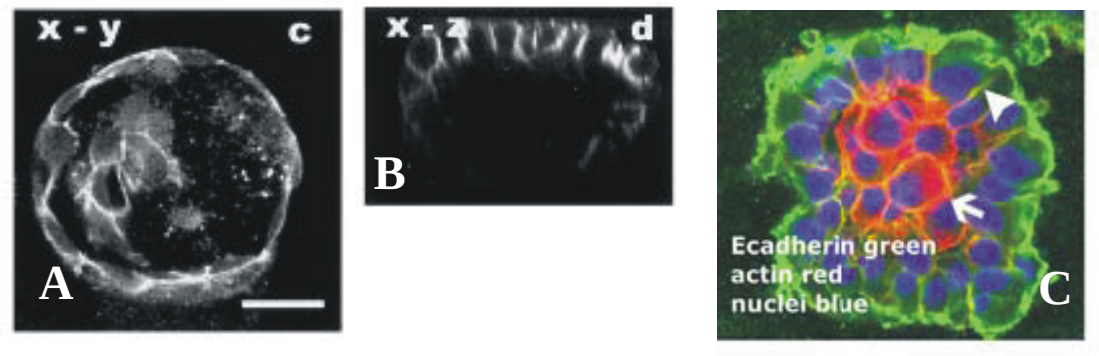


Figure 14 *Photobleaching of the immunofluorescence signal by using microscopes with different scanning speeds. Panels A and B show the distribution of E-cadherin in ts-src MDCK cells at permissive temperature. B is the z-section. Bleaching of the sample is noticeable in B. Bleaching affects the image obtained and some data may be lost. In C, E-cadherins in green, actin red and nucleai blue. The A and B E-cadherin staining is faint in the cells without matrix contact and with notable bleaching, especially in z-sections there appears to be no E-cadherin staining inside the cyst. Therefore an assumption that there was a lumen inside the cyst was easily made. The images are from I-II.*

Concerning bleaching, we noticed that working with a confocal microscope with slow scanning speed, led to considerable bleaching of the specimen. The problem of bleaching could be overcome by using a microscope with a faster scanning speed. Also use of double-or triple staining and their simultaneous recording instead of multiple single stainings and scannings proved to be vital to avoid bleaching.

In the publication I, only single immunostainings were used. Figure 14 A and B shows an example of how a low scanning speed affects the quality of the results (I).

Due to the harmful and deleterious effects of application of peptidases, several modifications were tested to enable sufficient penetration of antibodies without using proteolytic treatments. For that purpose we resorted to the setup 2 as described above.

The results show that when growing the cells on top of a thin ECM gel (Setup 2) immunostaining without prior treatment with peptidases could be introduced. On the other hand, when the cell clusters and cysts were grown on top of the gel, there was a risk of them being washed away during the immunostaining protocol. Despite its disadvantages, the setup 2 was used for testing for mitochondrial function. For multiple consecutive stainings, special care had to be taken to avoid flushing away the cells (II-III).

In order to guarantee mechanical support and favorable immunolabeling conditions, the technique of Debnath and Brugge (2003) method was modified. The cells were seeded in a thinner layer of matrix in smaller volumes (30 µl) on reusable FlexiPERM chambers. This technique enabled the culture of cells on top of a cover slip. This helped to avoid transferring the fragile gel for immunostaining and imaging. Thus, less material was lost during the protocol. Use of peptidases was not needed. Secondary fixation after immunostaining was also introduced in order to guarantee better preservation of the samples for imaging (II-III).

5.1.3 Immunofluorescence microscopy of living cells (II-III)

For some experiments (measurement of mitochondrial function and culture of ts-src MDCK cells transfected with pN1-ECad-EGFP), monitoring of living cells was required. The setup 2 was used for the mitochondrial activity measurements despite of its evident disadvantages of not providing proper mechanical support.

Because the mitochondrial function of the cell cultures were monitored every 24 hrs, it was important, however, that the setup enabled Mito-orange and Mito-green to be applied and washed away and the same cultures to be reused for similar experiments on the next day (II-III).

The ts-src MDCK cells transfected with a stable construct of pN1-ECad-EGFP permitted the continuous monitoring of the distribution of cadherin in living cells. Comparison of several setups showed that only setup 3 provided the proper matrix support and promoted growth of 3D structures. The cells were seeded in a thin gel and monitored with a video microscope every 20 min allowing the process of cyst formation to be monitored in the course of time (III).

5.2 Growth and supracellular organization of MDCK and ts-src MDCK cells in 2D and 3D cultures (I-III)

It is known from previous studies that the composition and physical properties of the surrounding growth matrix has an effect on the polarization of cells in 3D-culture (Discher et al., 2005; Yamada et al., 2007). Based on this, we modified the composition of the growth matrix in order to see, what kind of an effect composition and the density of the matrix has on the polarization process of MDCK cells. We specially monitored the changes in the distribution of junctional proteins and in the cyst formation in response to distinct changes in the composition and density of the matrix.

In 2D cultures the cells were grown on a cell culture dish.

In 3D experiments the following types of gels were used as representatives of different tissue types and densities:

1. Collagen I (high density)
2. Mixture of collagen (75%) and Matrigel (25%) (medium density)
3. Matrigel (low density)

The experiments were done separately by using MDCK and ts-src MDCK cells at various temperatures.

Furthermore, we wanted to distinguish the effect of deprivation of cell attachment on polarization. This was achieved by culturing the cells on a Petri dish in the presence of an antibody against $\alpha 2\beta 1$-integrin. Binding of this antibody to the integrin receptor prevents the cells from attaching to the matrix and forces them to grow in suspension (Torkko et al., 2008).

5.2.1 Cell polarity and cyst formation in MDCK cells in 2D and 3D culture (I-III)

In 2D, the MDCK cells were seen to form a well polarized monolayer of cells. The cells attached at their basal side to the cell culture dish. The apical side was facing towards the cell culture medium. Well-developed cell-cell adhesions along the lateral walls were seen to hold the neighboring cells together as judged by immunofluorescence microscopy. A schematic representation of a polarized monolayer of MDCK cells in 2D is shown in Fig. 14.

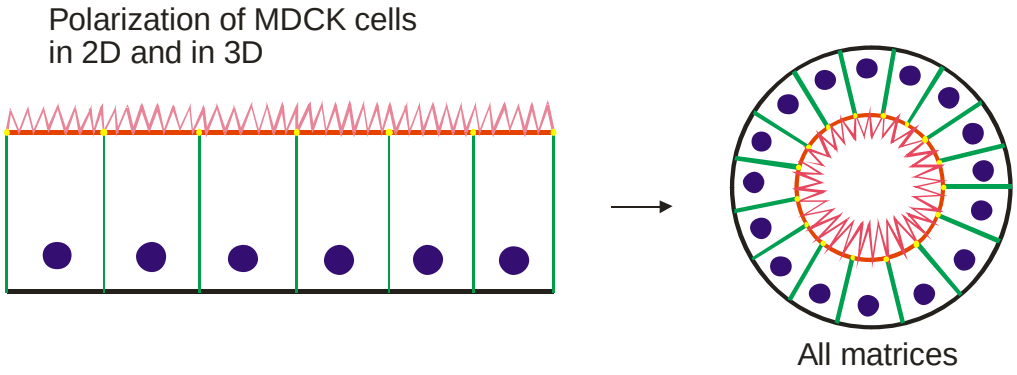
Previous studies have shown that MDCK cells in 3D cultures form a spheroidal cyst in which there is peripheral single-cell layer of polarized cells and a hollow cavity in the center. In the peripheral cells, complete polarization is observed. Lumen formation has been seen in all matrices tested (Wang et al., 1990, Martin-Belmonte et al., 2008).

In the present study, collagen I, mixture of collagen I and Matrigel and Matrigel alone were used as 3D culture matrices. In light microscopy, the cyst formation of MDCK cells grown in collagen and in Matrigel appeared very similar. This was verified by electron microscopy that showed a similar lumen formation and distribution of junctional components in both matrices. Based on these results, the subsequent experiments were carried out by using Matrigel-based matrix as growth medium.

Immunofluorescence microscopy was used to study the molecular and structural correlates of cell polarization and cyst formation of MDCK cells in Matrigel. Cadherin was seen along the lateral walls, where it co-localized with β-catenin and spectrin. Actin was seen along the lateral walls and delineating the lumen in apposition to microvilli. ZO-1 was located apically in point-like structures between the apical and basolateral cell walls.

There seemed to be non-vital cells trapped within the central lumen as judged by light and electron microscopy. As they appeared apoptotic by appearance, we used immunofluorescence microscopy with anti-caspase 3 antibodies to look at their state more closely. The results showed that there were caspase 3-positive apoptotic cells inside the lumen. The results suggest that the cells trapped inside the lumen without matrix contact were undergoing apoptosis. This observation is in line with the previous data showing that one of the mechanisms that contribute to lumen formation in MDCK cells in 3D cultures, and also in epithelial cells in general, is apoptosis of the centrally located, "trapped" cells, which in their new habitation are deprived of matrix contact. In the literature, such a mechanism of lumen formation is called hollowing (Wang et al., 1990; Martin-Belmonte et al., 2008; Debnath et al., 2003).

A schematic representation of a polarized MDCK cell cyst is shown in Fig. 15.



Cell	2D	3D	Junctions	Apoptosis
MDCK	Polarized monolayer	Polarized epithe-lium central lumen	Normal junctions zo-1 apically	Inside lumen in suspensions

Figure 15 *The polarization of MDCK in 2D and in 3D culture conditions.*

A second marker of apoptosis, the metabolic index, was measured by analyzing the mitochondrial activity of the cells. For that purpose, Mitotracker Green incorporation into the cells was used as a measure of mitochondrial mass and Mitotracker Orange incorporation as a measure of mitochondrial mass with oxidative capacity. Their ratio was called the metabolic index. There was a direct correlation between high metabolic index and triggering of apoptosis in normal cells. The higher their ratio the more apoptotic cells in the area in which the incorporation was measured.

In MDCK cells the metabolic index was higher for the first two days, but then decreased on day 3. This coincided with the formation of a central cavity in the cyst. Thus, together with the observations on cleaved caspase 3-positive cells in the central cavity clearly suggest that cavity formation in the cyst is due to the apoptosis of the cells inside the cell cluster. The data suggest that the formation of the cavity in MDCK cells is due to apoptosis of the cells without matrix contact.

5.2.2 Cell polarity and cyst formation in ts-src MDCK cells in 3D culture at non-permissive temperatures (I, III)

Next we wanted to determine the effect of culture conditions on the polarization of ts-src MDCK cells at non-permissive 40.5 ^{o}C temperature. For that purpose, the ts-src MDCK cells grown at non-permissive temperature in 2D, 3D and in suspensions. Their polarization and growth properties were compared to normal MDCK cells. The experiments in 3D were carried out in Matrigel.

In 2D-cultures, at non-permissive temperature, ts-src-MDCK cells formed a well-polarized monolayer identical to that of MDCK cells. As judged by immunofluorescence microscopy, the cell-cell junctions appeared to be well formed and tight junctions sealed the cells together apically. The distribution of junctional proteins resembled that of the MDCK cells grown in 2D.

In 3D cultures in Matrigel, when grown at non-permissive temperatures, ts-src MDCK cells formed cysts, as judged by immunofluorescence microscopy. The cysts appeared identical to cysts formed when MDCK cells were grown in 3D. Typically, the cysts had one central lumen and the cell-cell junctions were well formed. Cadherin and β-catenin were co-localized along the lateral walls. Some actin was seen along lateral walls, while the most prominent staining was seen in the apical region. ZO-1 was found at the border of the apical and lateral cell walls. This distribution of the polarization-related proteins was similar to that seen in normal cells in Matrigel.

Within the lumen of the cyst, some scattered cells were seen which, by their morphological features, were clearly different from the polarized epithelial cells of the "shell" of the cyst. Immunofluorescence staining showed that they were positive for cleaved caspase 3 indicating that they were undergoing apoptosis.

The metabolic index of the cells remained low and only started to rise on day 3. This coincided with stage of the growth of the spheroid when it had a sizable central cell mass. At this stage, the centrally located cells also stained positive for caspase 3.

Summary of the effect of culture conditions on ts-src MDCK cells at non-permissive temperature is shown in Fig. 16.

These results show that in the cyst formation in ts-src MDCK cells at non-permissive temperature in 3D Matrigel the peripheral cell layer undergoes normal polarization and differentiation probably due to interactions with the surrounding matrix. The lumen, on the other hand, seems to be shaped via apoptosis of the cells in the center of the cell cluster among the cells that lack direct contact with the matrix. Furthermore, apoptosis of the centrally located cells suggest that the cells do not have a default-like "polarity

program" but, instead, are dependent on matrix contact to initiate it. The distribution of junctional proteins and morphology of the cyst was identical to that seen in MDCK cells.

5.2.3 Cell polarity and cyst formation in ts-src MDCK cells in 2D and 3D culture at permissive temperature (I-III)

Next we wanted to analyze the effect of activated src on the polarization and cyst formation of ts-src MDCK cells in 2D and in 3D. For that purpose the cells were grown at permissive temperature (35 ^{o}C). For 3D-cultures, the following conditions were applied:

1. Collagen I (high density)
2. A mixture of collagen IV, laminin and heparan sulphate proteoglycans (medium density)
3. Collagen I and laminin (medium density)
4. Matrigel (low density)

The polarization and cyst formation were monitored and evaluated by immunofluorescence techniques by using antibodies as described above. Apoptosis was measured with antibodies against cleaved caspase 3 and by determining the metabolic index.

First, ts-src MDCK cells were grown at permissive temperature in 2D. This was done to compare the growth properties of transformed MDCK cells with those of non-transformed MDCK cells in 2D.

Immunofluorescence microscopy with antibodies against E-cadherin, ZO-1 and labeling with phalloidin showed that, instead of a well-polarized monolayer, seen in untransformed cells, transformed src MDCK cells formed a layer of cells with fibroblast-like morphology as shown schematically in Fig. 16. Loss of polarity was indicated for instance by weaker or absent staining for E-cadherin, β-catenin and ZO-1 cell-cell junctions (Fig. 16), publication II. These results showed that activation of src brings about a major change in cell morphology of MDCK cells, mostly characterized by loss of vertical polarity.

The major phenotypic features of the transformed MDCK cell monolayer in 2D are shown schematically in Fig. 16.

Next, ts-src MDCK cells were grown in 3D collagen I at 35 ^{o}C. They showed a growth pattern and morphology that was completely different from that of their normal counterparts. First, they did not show any cyst formation, but, instead, grew in clusters, in which the cells were loosely attached together. Second, there was no distinct cavity formation. Third, they showed no signs of polarity as indicated by lack of any sign of well-developed junctional complexes. Fourth, the cells displayed a motile phenotype as evidenced by numerous cellular protrusions. Fifth, there was no sign of apoptotic cells in the center of the cell clusters as studied by anti-caspase immunofluorescence microscopy. All in all, upon induction of src activity in in 3D collagen, ts-src MDCK cells at 35 ^{o}C seemed to have a similar non-polarized phenotype as in 2D. They also failed to form the cyst-like structures with a central cavity and the peripheral layer of polarized cells.

When compared to growth in collagen I, the cells in Matrigel showed a totally different phenotype. Two distinct populations within the cyst were observed. First, the cells in contact with Matrigel showed signs of polarity as judged by immunofluorescence microscopy. In them, cadherin and β-catenin as well as fodrin were localized along the lateral walls. The second population of cells was seen within a cyst. They looked mesenchymal and showed no signs of polarity. In them, actin staining was prominent. On the other hand, less E-cadherin and β-catenin was seen in comparison to normal cells.

In ts-src MDCK cells at non-permissive temperature in Matrigel, there were scattered cells in the lumen that were apoptotic. Thus, we next wanted to see whether the cells in the central area of the cysts also showed signs of apoptosis. Therefore, we used staining for caspase 3 and measurement of the metabolic index.

There were no caspase 3-positive cells in the cysts of transformed ts-src MDCK cells in Matrigel indicating that the cells in the central area are not undergoing apoptosis. This was confirmed by the studies on the metabolic index. It showed that the ts-src MDCK cells at permissive temperature had a higher metabolic index than normal MDCK cells, but showed no signs of apoptosis.

The above results showed that the ts-src transformed MDCK cells behaved quite differently in collagen I-based matrix and in Matrigel.

In order to explore further the effect of the matrix composition and density, we next studied the same cells in different matrices in which the components of basal lamina such as collagen IV, laminin and proteoglycans were added to the collagen I-based matrix. In contrast to Matrigel, which represents low density matrix and collagen I that represents high-density matrix, these variants of collagen I-matrices belong to medium-density matrices.

In all the matrices in which basal lamina component laminin was added to collagen I matrix, distinctly more organized. Cell clusters were seen than in pure collagen I matrix. Most distinctly, no mesenchymal looking cells with a motile phenotype were seen as was common in collagen I. Immunofluorescence studies and metabolic index determinations showed that the cells in the clusters were phenotypically quite homogeneous. There were neither signs of polarity in the outermost cell layer nor any signs of apoptosis in the centrally located cells.

The above results showed that the transformed ts-src MDCK cells displayed in 3D quite different phenotypes and quite distinct supracellular organizations depending on the matrix they were grown in. The most distinct differences were seen when comparing the cells grown in collagen I and in Matrigel –matrices. While in collagen I the cells displayed the major phenotypic features of transformed cells (lack of polarity, motile phenotype) that were also seen when the same cells were grown in 2D, in Matrigel they looked “normalized” as evidence by polarization of the outermost cell layer, lack of motile phenotype and lumen formation by apoptosis. Overall, Matrigel seemed to have a counteracting effect on the development of transformed features that were fully in display in collagen I. However, it is noteworthy that, in contrast to normal MDCK cells and nontransformed ts-src MDCK cells in Matrigel, transformed cells in Matrigel were not seen to form a complete cavity in their center. Testing of several basement membrane components together with collagen I showed that none of them (laminin, collagen IV and

proteoglycans) was able to restore, when added to collagen I matrix, the morphology seen in cells grown in Matrigel. Thus, components of Matrigel other than basement membrane proteins should be considered as the active components accounting for the differences between collagen I and Matrigel matrices.

Overall, these results suggested that differences in the matrix composition greatly affects the way the src-induced changes become manifest in ts-src MDCK cells in 3D.

The effects of matrices of different compositions and densities on transformed ts-src MDCK cells are shown in Fig. 16.

5.2.4 The effect of src-kinase inhibitor pp2 on ts-src MDCK cells at permissive temperature in 2D and in 3D Matrigel (III)

In order to determine whether the changes seen in morphology of ts-src MDCK cells were src-kinase specific, an experiment involving the use of the src-kinase inhibitor pp2 was designed. The ts-src MDCK cells at permissive temperature were used as a control.

The effect of pp2 on ts-src MDCK cells in 2D cultures are described in Palovuori et al. (2003). It was shown that, at permissive temperature, in the presence of pp2, the ts-src MDCK cells formed a monolayer of polarized cells with cell-cell junctions and cuboidal shape. This phenotype is similar to that of the ts-src MDCK cells grown at non-permissive temperature (Palovuori et al., 2003). Thus, based on these studies, it can be concluded that the distinct phenotypic changes seen in ts-src MDCK cells when they are switched from non-permissive to permissive temperature are caused by src activity. The effect of pp2 on ts-src MDCK cells is shown in Fig. 16.

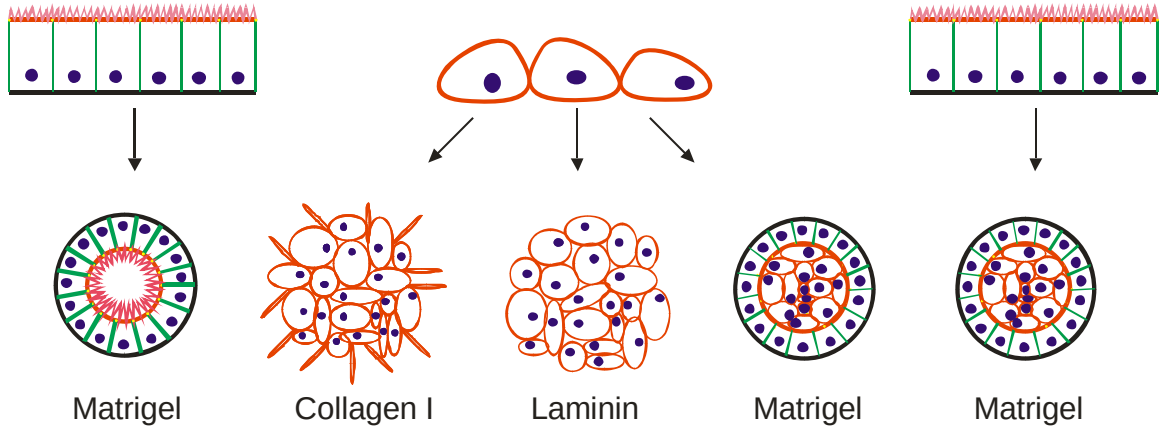
Next we studied the effect of pp2 to the phenotypic features of ts-src MDCK cells grown in Matrigel.

In 3D Matrigel, ts-src MDCK cells at non-permissive temperature showed a cyst-like structure with a central lumen as described above. At permissive temperature, a cyst-like structure with mesenchymal looking cells in the center and polarized epithelial cells in the periphery were seen, as described in 5.2.3. In the presence of pp2, the cell clusters retained the same organization as seen without pp2. There were still vital cells in central cell mass and no cytological signs of apoptosis among these cells were seen.

The phenotypic features of ts-src MDCK cells at 35 ^{o}C in the presence of pp2 were studied also by using immunofluorescence microscopy and markers of polarity and apoptosis. Cadherin, for instance, was seen along the lateral walls of the peripheral, more epithelial looking cells. The mesenchymal-looking cells in the central area were characterized by high content and distinct organization of actin fibers. On the other hand, none of the cells in the lumen showed apoptotic markers. Thus, also on the basis of these molecular markers for cell polarity, it can be concluded that, in the presence of pp2, ts-src MDCK cells at permissive temperature retain the polarity of the peripheral cell layer. But it does not lead to activation of apoptosis, which seems to be lost or counteracted in luminal cells in ts-src MDCK cells at permissive temperature even in the presence of src inhibitor pp2.

The results suggest that src-kinase inhibitor pp2 has different effects in 2D and in 3D. In 2D cell cultures it was shown to induce apoptosis in transformed cell lines and inhibit the disruption of adherens junctions in ts-src MDCK cells (Palovuori et al., 2003) as if reversing or blocking the effects of active src. In 3D, pp2 did not have an effect on cell polarity suggesting that, in Matrigel environment, either, (1) no src-specific effects on polarity are seen in this cell compartment, or (2) that there are src-specific effects that are, however, counteracted by stimuli from Matrigel. Interestingly, presence of pp2 could not restore the apoptotic phenotype in the central cells although previous results suggested that the absence of apoptotic cells in the transformed ts-src MDCK cells is due to specific effect of src activation.

The polarization and transformation processes seemed to be different in 2D and 3D culture conditions and therefore specific kinase inhibitors may have distinct effects in 2D, in 3D and *in vivo* models. The findings are in line with other data showing that the data gathered from 2D cannot be generalized to 3D, only 3D matrices give proper cues for cell lines to form the spherical structures seen *in vivo* (Pampaloni et al., 2007; Yamada et al., 2007).



Src MDCK	2D	3D	Junctions	Apoptosis
Nonpermissive Matrigel	Polarized monolayer	Polarized epithelium central lumen	Normal junctions zo-1 apically	Apoptosis inside lumen, and in suspensions
Permissive Collagen I	Nonpolarized monolayer	No polarity invasive protrusions	Poor junctions, no polarity	No apoptosis
Permissive Laminin	Nonpolarized monolayer	No polarity	Poor junctions, no polarity	No apoptosis
Permissive Matrigel	Nonpolarized monolayer	Lumen filled with cells	Better junctions with matrix contact	No apoptosis, little in suspensions
Permissive Matrigel + pp2	Polarized monolayer	Lumen filled with cells	Better junctions with matrix contact	No apoptosis, little in suspensions

Figure 16 *The effect of matrix on ts-src MDCK cells in various conditions.*

As a conclusion, it was shown that the effect of src-kinase inhibitor pp2 has different effects on the cell morphology in 2D and in 3D. In 2D it inhibits the deleterious effect of src on adherens junctions and restores cell polarization. In 3D in Matrigel, it has no effect on the morphology of ts-src MDCK cells.

5.2.5 Suspension culture - Blocking cell attachment via integrin blocking antibody (III)

The results from the experiments above suggested that the cavity formation in the cell cyst of MDCK cells is due to apoptosis. Since apoptosis is seen in central cells that are not in contact with the matrix material, it can be hypothesized that the apoptotic process could be due to loss of matrix contact when some of the cells in the expanding cluster of cells become trapped in the center of the cell cluster.

Next we wanted to elucidate whether the MDCK cells are sensitive to such a loss of growth subratum contact. This was explored by forcing the cells to grow in suspension. It was achieved by growing the cells in the presence of antibodies to $\alpha 2\beta 1$-integrin. This antibody binds to the integrin and prevents the binding of the cells to collagen I. This, in turn, leads to the detachment of the cells from their culture substratum. Apoptotic cells were detected by using FITC-Annexin binding assay. The metabolic index of the suspension culture cells was also measured as described before.

The cells were grown in suspensions for 12 to 24 hrs prior to determining the degree of apoptosis by counting the number of apoptotic cells. In order to detect whether matrix contact can rescue the cells from apoptosis, the cells were replanted in Matrigel after 24 hrs in suspension.

The results showed that:

1. 25 % of MDCK cells underwent apoptosis when grown in suspension. Re-establishment of matrix contact could not rescue already apoptotic cells. However, the cells showing no signs of apoptosis in suspension were capable of forming a normal cyst with a central lumen after reimplantation in Matrigel.

2. 100 % of ts-src MDCK cells grown at non-permissive temperature underwent apoptosis within 24 hrs. Reattachment to Matrigel did not rescue the cells from apoptosis.

3. 8 % of the ts-src MDCK cells at permissive temperatures went into apoptosis when grown in suspension. After re-establishment of matrix contact to Matrigel, the cells were able to form cysts with viable, mesenchymal looking cells inside the lumen.

4. 5 % of the ts-src MDCK cells at permissive temperatures in the presence of pp2 went into apoptosis when grown in suspension. The cells were able to form similar cysts in Matrigel as the cells without pp2 with viable cells filling the lumen.

As a conclusion, normal MDCK cells are able to survive when deprived of matrix contact. That may partly be due to their ability to secrete their own ECM proteins as shown by Wang et al. (1990). Ts-src MDCK cells at non-permissive temperatures, on the other hand, require matrix contact for their survival since they all seemed to succumb to apoptosis when grown in suspension. In stark contrast to this, the ts-src MDCK cells

grown at permissive temperatures seemed to evade apoptosis even when deprived of matrix contact.

As a general conclusion, these results show that the differences in the cavity formation in ts-src MDCK cells at non-permissive and permissive temperatures could be due to different sensitivities of nontransformed and src-transformed cells to apoptosis induced by loss of contact to matrix.

5.2.6 Dynamics of establishment and maintenance of cell polarity, lumen formation and cell-cell junctions in ts-src MDCK cells in 3D (III)

Activation of v-src by temperature shift provides a unique opportunity to monitor the changes that are associated with the transformation process of ts-src MDCK cells. By using the optimized immunofluorescence techniques it also makes it possible to investigate how the ts-src MDCK cells are organized as a cyst and how the lumen is formed and how src-transformation alters the polarized structures in 3D.

In the experiments described above, we looked at final structural organization of cells grown at constant conditions, with src was either active or inactive. In the follow-up of such experiments, we wanted to look further into the dynamics of the process and to investigate the mechanisms by which activation and inactivation of src affects the cells in 3D culture conditions. For that purpose we used immunolabeling and immunofluorescence microscopy. After temperature shift, the cells were immunolabeled at certain time points and then scanned with the confocal microscope.

In one set of experiments, ts-src MDCK cells were first cultured at permissive temperature in Matrigel for 5 days. Thereafter, src was inactivated by changing the cells to non-permissive temperature. In another set of experiments, ts-src MDCK cells were first grown at non-permissive temperatures in Matrigel for 7 days. After that the cultures were switched to permissive temperature. The cells were either immunolabeled with antibodies to E-cadherin and or treated with fluorescent-labeled phalloidin, a reagent binding to filamentous actin.

In the first set of experiments, starting at permissive temperature, the cells made a cyst with vital, mesenchymal looking cells filling the lumen. After inactivation of src by a shift to non-permissive temperature, a small lumen started to appear in the center within 1 hr. After 24 hrs the lumen grew larger. Parallelly, emergence of apoptosis of the cells in the area of the emerging lumen was seen with subsequent clearance of apoptotic bodies inside the lumen. Within a couple of days the inner cell population underwent apoptosis and the apoptotic bodies were cleared from the lumen.

In the second set of experiments, starting at non-permissive temperature, the cells formed a cyst with central lumen. Peripheral cells showed signs of polarity, actin was found apically and cadherin laterally. Within few hours of the shift to permissive temperature, disassociation of adherens junctions and rounding of the cells was seen. Later on, the central lumen became filled with dividing cells.

Figure 17 D-F shows a schematic representation of phenotypic features in activation of src of in 3D.

The results from immunofluorescence microscopy were confirmed with the pN1-ECad-EGFP ts-src-MDCK cell line, where the cell culture can be monitored in real time with a video microscope. The results are discussed later in chapter 5.2.9.1.

5.2.7 Cadherin recycling in 2D (II)

Cell-cell junctions are in a key role in the establishment of cell polarity. Junctional proteins are continuously recycled to the plasma membrane from the cytosol and back. That enables the rapid changes in cell morphology and response to various stimuli (Yap et al., 2007).

Src-induced transformation has a dramatic effect on cell junctions. Activation of src is accompanied by disruption of E-cadherin –based adherens junctions and internalization of E-cadherin into the cytosol (Behrens et al., 1993, Palovuori et al., 2003).

We wanted to learn about how the src-transformation affects endocytosis and intracellular transport of E-cadherin in 2D. For that purpose the cells at non-permissive temperature were grown to confluency, then kept on ice and incubated with an antibody against the cytoplasmic tail of E-cadherin (rr1) anti E-Cadherin antibody.). The cells were then transferred to 40.5 ^{o}C or 35 ^{o}C to allow internalization of the cadherin.
The cells were also stained for β-catenin to determine the dynamics of the E-cadherin-β-catenin complex and to investigate whether the complex was disassembled. Colocalization analysis of internalized E-cadherin with β-catenin was carried out using the Olympus FluoView1000 software.

The results showed that internalization of E-cadherin took place both at non-permissive and permissive temperature, but it was more efficient at non-permissive temperature.

When the cells were fixed on ice at time point 0 min, rr1 staining was seen on the cell surface, mostly at the edges of cell islets.

In 30 min after warming the cells either to 40.5 ^{o}C or 35 ^{o}C, rr1-labelled E-cadherin was seen in the cell interior in apical vesicles occasionally devoid of β-catenin. Along the basal surface, however, it co-localized with β-catenin.

The results indicate an efficient internalization of cadherin taking place. 70 % of such vacuoles were not stained for β-catenin, indicative of disassembly of β-catenin /cadherin complex upon endocytosis. However, a distinctive colocalization of β-catenin and cadherin was seen along the basal surface of the cells. At 30 min after internalization co-localization coefficients were higher at 35 ^{o}C suggesting that at permissive temperature AJs are disintegrating and both proteins are internalized together.

From 60 minutes on incubation at 40.5 ^{o}C, rr1-labelled E-cadherin was seen partially in apical vesicles and partially accumulated back to lateral membranes. Co-localization coefficient for E-cadherin in apical vesicles was very low at 40.5 ^{o}C suggesting that they belong to the clathrin-mediated recycling route where catenins are released from E-cadherin upon internalization. In the cells incubated 60 min at 35 ^{o}C only a thin cadherin layer was found at lateral membranes and the majority remained in the cytoplasm and in the apical vesicles devoid of β-catenin.

Collectively, impaired recycling of E-cadherin in src-transformed MDCK cells could be at least partially responsible for the defective establishment/maintenance of cell-cell junctions as seen in ts-src MDCK cells grown at permissive temperature in 3D.

5.2.8 The role of TGFβ1 and anti- TGFβ on lumen formation (I)

The results from the reconstitution experiments described in 5.2.3, showed that the basement membrane components that are present in Matrigel but not in collagen I matrix could not account for the differences seen in cyst formation in ts-src MDCK cells grown at permissive temperature in these two matrices. Thus, we hypothesized that the Matrigel-specific effect on src-transformed cell could be due to soluble factors instead of insoluble stromal components. Indeed, based on the results of several previous studies it has been suggested that the major factor in Matrigel that underlies its polarization promoting capacity is TGFβ (Kuzuya and Kinsella 1994).

In order to study the role of soluble growth factors, we first grew ts-src MDCK cells at permissive temperature in growth factor depleted-Matrigel, *i.e*. in Matrigel which lacks e.g. Epithelial growth factor (EGF), Insulin like growth factor -1 (IGF-1) and platelet derived growth factor (PDGF). Under these conditions the cells did not from a lumen as was seen in "regular" Matrigel. Instead, they formed structures very similar to those grown in a mixture of collagen I supplemented either with laminin or a mixture of collagen type IV, laminin and proteoglycans. This showed that even an ECM rich environment, which is devoid of soluble growth factors, is not sufficient for the proper differentiation of ts-src MDCK cells in 3D.

As a next step, we tested the effect of TGFβ1 on src-transformed MDCK cells grown in growth factor depleted Matrigel. A partial rescue of the polarized phenotype and lumen formation was seen when TGFβ was added to Matrigel. The effect was concentration dependent. The effect was inhibited with anti-TGFβ1 antibodies. Thus, it was concluded that bypass or prevention of the effect of ongenically active src on cell polarity and cyst formation could be assigned, at least partially, to TGFβ1. It was noteworthy; however, that soluble TGFβ1 alone had not such an effect. This suggests that not only TGFβ1, but also its binding and accessibility to cells in an appropriate matrix context is required.

5.2.9 Dynamics of cyst formation in ts-src MDCK cells at non-permissive and permissive temperatures as revealed by microscopy of living cells (III)

Fluorescent pN1-ECad-EGFP ts-src MDCK cell line (III)

In order to obtain a more dynamic view of the polarization and transformation process of ts-src MDCK cells, traceable markers and real time monitoring of the events is needed. For that purpose, a ts-src MDCK cell line constitutively expressing E-cadherin molecule

tagged with GFP was created. The ts-src MDCK cells were stably transfected with the construct pN1-ECad-EGFP and used for further studies.

For live microscopy, the pN1-ECad-EGFP ts-src-MDCK cells were mixed into Matrigel and were grown in an Okolab cell culture chamber. The cultures were examined every 20 min under Olympus CellP video microscope equipped with a 60x water immersion objective.

First, in order to monitor the dynamics of E-cadherin during the polarization process, the transfected cells were cultured at permissive temperature for three days. In the cell clusters, a central lumen filled with loosely arranged viable cells were seen from day 2 to 3 onwards. E-cadherin was seen at the cell walls, but the cells inside the cyst showed only a weak signal.

Next, the temperature was switched to non-permissive and the cells were kept under those conditions for three days. Continuous monitoring at 20-min intervals showed that the cells inside the lumen without matrix contact lost their cadherin signal in two days. On the other hand, the cells along the periphery and with matrix contact showed more intensive cadherin signal along the lateral cell walls. Immunostaining with antibodies against cleaved caspase 3 showed that the cells inside the cyst were undergoing apoptosis.

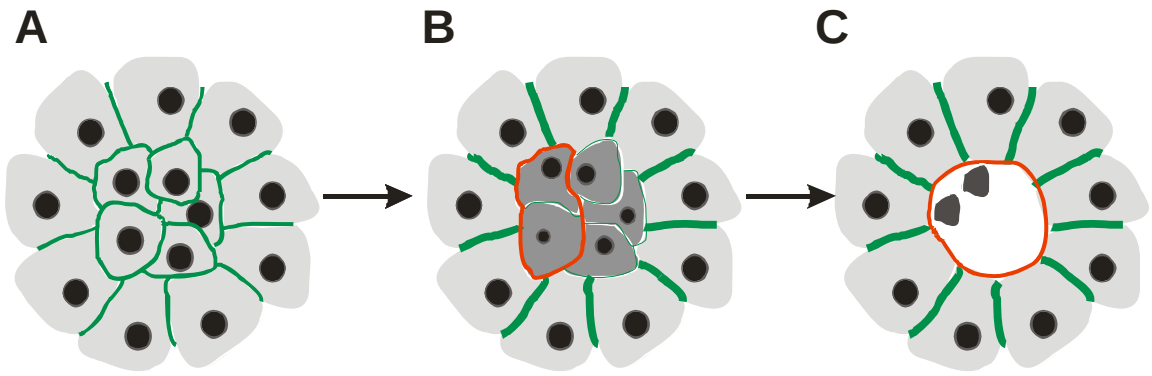


Figure 17 *A) The figure 17 A-C shows the dynamics of cadherin and cyst formation when src is inactivated in 3D by using the pN1-ECad-EGFP ts-src MDCK cells and immunostainings of ts-src MDCK cells. A) Shows the cell cluster of ts-src MDCK cells at permissive temperature. The lumen is filled with vitals cells. B) When the temperature is changed to non-permissive, the cells without matrix contact lost their cadherin signal and appeared to be apoptotic. In immunostaining of cadherin and actin, a small lumen appears in few hours. C) In three days cadherin is seen on the lateral walls of a well polarized cell cyst. The remnants of the apoptotic cells were seen inside the central lumen, in immunostaining actin delineates the lumen and the apoptosis is confirmed by antibodies against cleaved caspase 3.*

Next, in order to monitor the dynamics of the transformation process induced by src activation, the following experiment was performed. The culture was started by keeping the cells at non-permissive temperature and the cells were allowed to polarize. Then the temperature was switched to permissive. Upon continuous monitoring at 20-min intervals, a rapid disintegration of E-cadherin-based adherens junctions of polarized ts-src MDCK cells was seen. At first, the cadherin signal became indistinct and, later, two separate closely spaced plasma membrane domains could be seen as a sign of separation of the

cell-cell junctions into two halves, each in a separate cell. Within hours, the cells were further separated and finally became rounded. In these cells only a weak cadherin signal was seen.

Alongside with the changes in the cell-cell junctions, there were also distinct changes in central cell mass. Upon switch from permissive to non-permissive temperature the cells without matrix contact underwent apoptosis and a cyst with a central lumen was formed. Upon switching from non-permissive to permissive temperature on the other hand, the lumen was refilled with vital cells with a mesenchymal phenotype (III).

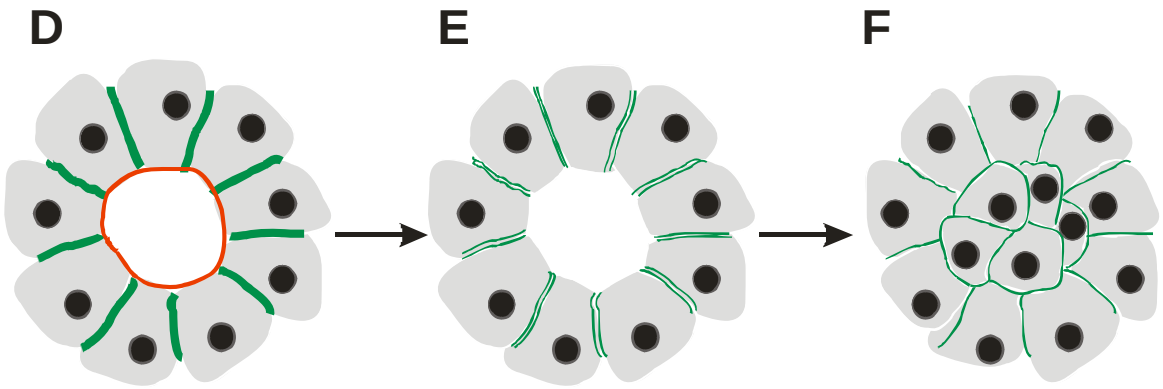


Figure 17 *B)Figure 16 D-E shows the dynamics of cadherin and cyst formation when src is activated in 3D by using the pN1-ECad-EGFP ts-src MDCK cells and immunostainings of ts-src MDCK cells. D) The ts-src MDCK cells grown at non-permissive temperature show signs of polarity. E-cadherin is located along the lateral walls of a polarized epithelium and there is central lumen inside the cyst. E) When src is activated, the adherens junctions disintegrate rapidly, the neighboring cells detach from each other. E-cadherin signal seems to first become more indefinite before the two cell walls separate from each other and two signals can be seen. F) In days the activation of src leads to the filling of the lumen by dividing cells.*

The results of these studies show that, in Matrigel, both at permissive and non-permissive temperatures, a fairly orderly polarization, which correlates with the establishment and maintenance of cadherin based cell-cell junctions along the lateral walls of the matrix-facing “cortical” cell layer, was seen. On the other hand, loss of polarity was seen in the centrally located cell mass, which was maintained in the luminal area as a mesenchymal-looking cell population at permissive temperature and that was depleted, via apoptosis, leaving a central lumen, at non-permissive temperature (III).

Mitochondrial function and apoptosis (II-III)

The results described above strongly suggested that the lumen formation in ts-src MDCK cells at non-permissive temperature is due to apoptosis of the cells which form the central mass and which are not in contact with the matrix. On the other hand, at permissive temperature, no apoptosis of the centrally located cells was seen. This suggested that

apoptosis is important in the sculpturing of the hollow cavities in the cyst-like structures, which are delineated by a cortical layer of polarized cells. The importance of the loss of matrix contact as putative inducer of apoptosis was demonstrated in studies with cells grown in suspension.

In order to further verify the role of apoptosis in lumen formation, we decided to study mitochondrial function in normal and ts-src MDCK cells. For that purpose, incorporation of Mitotracker Green into the cells was used as a measure of mitochondrial mass and Mitotracker Orange as a measure of mitochondrial oxidative capacity. Their ratio, designated as the metabolic index, was taken to represent the inverse of the proportion of actively respirating mitochondria and, thence, to be directly correlated with the proportion of apoptotic cells (Buckman et al., 2001). The mitochondrial function and its relation to apoptosis were studied in MDCK and ts-src MDCK cells in suspension and in Matrigel.

The cells were grown in suspensions as described before. The MDCK cells were grown at 37 ^{o}C and ts-src MDCK cells were grown at non-permissive and permissive temperatures with or without src-kinase inhibitor pp2. The metabolic index was measured at 12, 15 and 24 hrs. In 3D Matrigel, MDCK cells were grown at 37 ^{o}C and ts-src MDCK cells were grown at non-permissive and permissive temperatures with or without src-kinase inhibitor pp2 and the mitochondrial function was measured daily for a week.

In suspension cultures, the normal MDCK cells showed a higher metabolic index than ts-src MDCK cells at permissive temperature. Src-kinase inhibitor pp2 did not have any effect on the index. At non-permissive temperature, where all the cells were apoptotic as shown by experiments, described by immunofluorescent microscopy, the index was highest.

The results from these suspension culture studies suggest that high metabolic index correlates directly with the extent of apoptosis. The index can be used as a measure of both the proportion of malfunctioning mitochondria and of cells undergoing apoptosis.

Next we studied the metabolic index in 3D culture conditions. We compared the metabolic indexes of MDCK cells to those of ts-src MDCK cells cultured under various conditions. The metabolic index was low in MDCK cells when compared to ts-src MDCK cells at each temperature. The ts-src MDCK cells grown at non-permissive temperature had the highest metabolic index. They showed signs of lumen formation from the day three and the cells inside the lumen without matrix contact showed signs of apoptosis. At permissive temperature there was not such a rise in the metabolic index and no apoptosis was monitored.

In order to determine if the change in mitochondrial metabolism was src-kinase specific, src- kinase inhibitor pp2 was added to the cultures. It had only a minor effect on the metabolic index and the number of apoptotic cells and the degree of lumen formation remained the same irrespective whether pp2 was present or not.

All in all, also the results on mitochondrial function strongly support the conclusion reached also by other means described above that the cavity formation in MDCK cells and in ts-src MDCK cells at non-permissive temperature is due to apoptosis in central cell mass that is devoid of matrix contact in the growing cell mass (II-III).

5.3 Effect of 2D-3D shift and src activation on 2D and 3D in gene expression (II-III)

Polarization of epithelial cells is a part of their differentiation program and, as such, a least partially based on genetic programming. We decided to explore what kind of genetic changes are associated with the polarization process.

The results presented above clearly showed that the polarization process and extent of apoptosis was different in 2D when compared to 3D cultures. On the other hand, shift from non-permissive to permissive temperature resulted in drastically different phenotype in ts-src MDCK cells. Adding to the complexity, the transformation-dependent changes were different depending on whether 2D or 3D-culture was applied. Thus, by elucidating the differences in gene expression upon transformation in 2D and in 3D, insight could be gained into genetic programs that regulate these events.

We used DNA microarray technology to detect the global changes in gene expression upon polarization and transformation both in 2D and in 3D. RT-PCR and Western blotting were used to verify the changes in the expression levels of the genes that showed the most distinct and consistent changes in gene array analyses.

5.3.1 Gene microarray-analysis of MDCK and ts-src MDCK cells grown in 2D and in 3D cultures (II-III)

The results described above showed that both in 2D and in 3D conditions epithelial phenotype prevailed in MDCK cells and in ts-src MDCK cells at non-permissive temperature. On the other hand, mesenchymal phenotype was seen in ts-src MDCK cells grown in 2D at permissive temperature Addition of pp2 reversed this and led to maintenance of epithelial phenotype.

In 3D at permissive temperature with or without pp2, ts-src MDCK cells showed a phenotype with polarized cells at the periphery of the sphere and mesenchymal cells filling the lumen.

In order to gain insight into the genetic changes associated with the growth and polarization in 3D as opposed to 2D and to the genetic changes associated with transformation in 3D and in 2D as opposed to normal growth in 2D and in 3D, the cells were grown under the following conditions:

1. MDCK cells in 2D and in 3D Matrigel at 35 ^{o}C and 40.5 ^{o}C
2. Ts-src MDCK cells at permissive temperature (35 ^{o}C) in 2D and in 3D Matrigel
3. Ts-src MDCK cells at non-permissive temperature (40.5 ^{o}C) in 2D and in 3D Matrigel
4. Ts-src MDCK cells at permissive temperature (35 ^{o}C) in 2D and in 3D Matrigel with pp2

The detailed protocols of the analyses are shown in Fig. 18. The changes in gene-expression profiles were analyzed with Affymetrix Gene Chip program. The results of the

comparison of gene expression under these conditions in 2D and in 3D are shown in Fig. 18.

1. *2D vs. 3D*

First we analyzed the alteration of gene expression upon shift from 2D to 3D. The gene expression profiles of normal MDCK cells grown at 35 ^{o}C and at 40.5 ^{o}C, of ts-src MDCK at 35 ^{o}C and at 40.5 ^{o}C in 2D and MDCK cells grown at 35 ^{o}C and at 40.5 ^{o}C and of ts-src MDCK in Matrigel at 35 ^{o}C and at 40.5 ^{o}C, all grown in 2D and in 3D, were pooled together. The genes that changed their expression level 5-fold up or down either due to temperature shift or due to activation of src were excluded from the results. The remaining genes were considered to be associated with the altered phenotype brought upon the shift from 2D to 3D.

The following conclusions could be drawn from the gene profiling analysis. Only a small number of genes (118) were altered upon a switch from 2D to 3D over 5 fold in each of the pairwise from-2D-to-3D comparisons as described in the preceding chapter. Most of these genes that were differently regulated encoded proteins involved in regulation of actin, cell signaling, apoptosis or vesicular transport. One of the genes most downregulated upon the 2D- 3D shift was survivin, that inhibits apoptosis and promotes cell division. Remarkably, no genes encoding for cytoskeletal proteins or cell-cell junctional proteins were among the differentially expressed genes. This suggested that, when compared to the morphological changes due to the shift from 2D to 3D, forming a monolayer in 2D or a cyst in 3D does not require transcriptional activity of such genes. This strongly suggests that the rearrangements seen e.g. in cell-cell junctions and in cytoskeleton are due to translational regulation of the corresponding mRNAs or due post-translational modifications or redistributions of the proteins themselves.

2. *Activation of src in 2D; comparison between MDCK and ts-src MDCK cells*

Next we wanted to look at the changes in gene expression associated with activation of src in 2D. The following comparisons were made: ts-src MDCK cells at permissive (35 ^{o}C) temperature were compared to normal MDCK cells at 37 ^{o}C, to ts-src MDCK cells at non-permissive (40.5 ^{o}C) and to ts-src MDCK cells grown in the presence of src-kinase inhibitor pp2 at permissive (35 ^{o}C) temperature, all grown in 2D. The alterations in gene expression that changed to the same direction over 5 fold were included in the results.

Remarkably, only a small amount of genes were altered; the genes downregulated were chemokine ligand 2 and Rab5. The expression of chemokine ligand 2 suggests that it could play an immunomodulatory role in transformation. Rab 5, on the other hand, could play a role in the altered vesicular transport events in transformed cells

3. *Activation of src in 3D; comparison between MDCK and ts-src MDCK cells*

Next we wanted to look at the changes in gene expression associated with activation of src in 3D. For that purpose, the following comparisons were made: ts-src MDCK cells at permissive (35 ^{o}C) temperature were compared to MDCK cells, ts-src MDCK cells at non-permissive (40.5 ^{o}C) and ts-src MDCK cells grown in the presence of src-kinase inhibitor pp2 at permissive (35 ^{o}C) temperature, all grown in 3D in Matrigel.

Altogether, 114 genes were up- or downregulated 5-fold or more in each of the pairwise comparisons. The most interesting of them were those encoding cytoskeletal proteins, proteins of vesicular transport, proteins of cell division and cell survival, proteins involved in mitochondrial function and some lysosomal proteins. The detailed list of the altered genes is shown in Fig. 18 (II).

4. *Activation of src in 2D; comparison between ts-src MDCK cells at non-permissive and permissive temperatures*

Next we wanted to find out the genes affected by activation of src in ts-src MDCK cells. In order to narrow down to src-transformation specific changes in gene expression, MDCK cells were excluded from the comparisons. The comparison of genetic profiles of ts-src MDCK cells were done at non-permissive and permissive temperature with or without pp2 was done.

First we looked at the genetic profiles associated with activation of src in 2D. For that purpose, ts-src MDCK cells grown in 2D at permissive (35 ^{o}C) temperature were compared to the same cells grown at permissive (35 ^{o}C) temperature with src-kinase inhibitor pp2 and with cells grown at non-permissive (40.5 ^{o}C) temperature. The changes over 5-fold were included in the study.

There were 90 genes that changed their expression level over 5-fold in each of the pairwise experiments. Changes were seen in genes encoding for cytoskeletal proteins e.g. ZO-1, for extracellular matrix proteins, for proteins involved in cell division and in immune response. A more detailed list of changes is shown in Fig. 19. (III)

5. *Activation of src in 3D; comparison between ts-src MDCK cells at non-permissive and permissive temperatures*

The genetic profiles of ts-src MDCK cells grown in 3D at permissive (35 ^{o}C) temperature were compared to the same cells grown at permissive (35 ^{o}C) temperature with src-kinase inhibitor pp2 or at non-permissive (40.5 ^{o}C) temperature.

192 genes were altered over 5-fold. Changes were seen in genes encoding for proteins involved in gene expression, in cell division, in inhibition of apoptosis, in cell metabolism, in mitochondrial function, in actin cytoskeleton and for mechanosensitive proteins.

The increase in the expression of survivin was observed when comparing the cells at non-permissive to the cells at permissive temperatures. Also upregulation of gene encoding PI3K was among the major changes seen in response to transformation by src. The most interesting changes are shown in Fig. 19. (III)

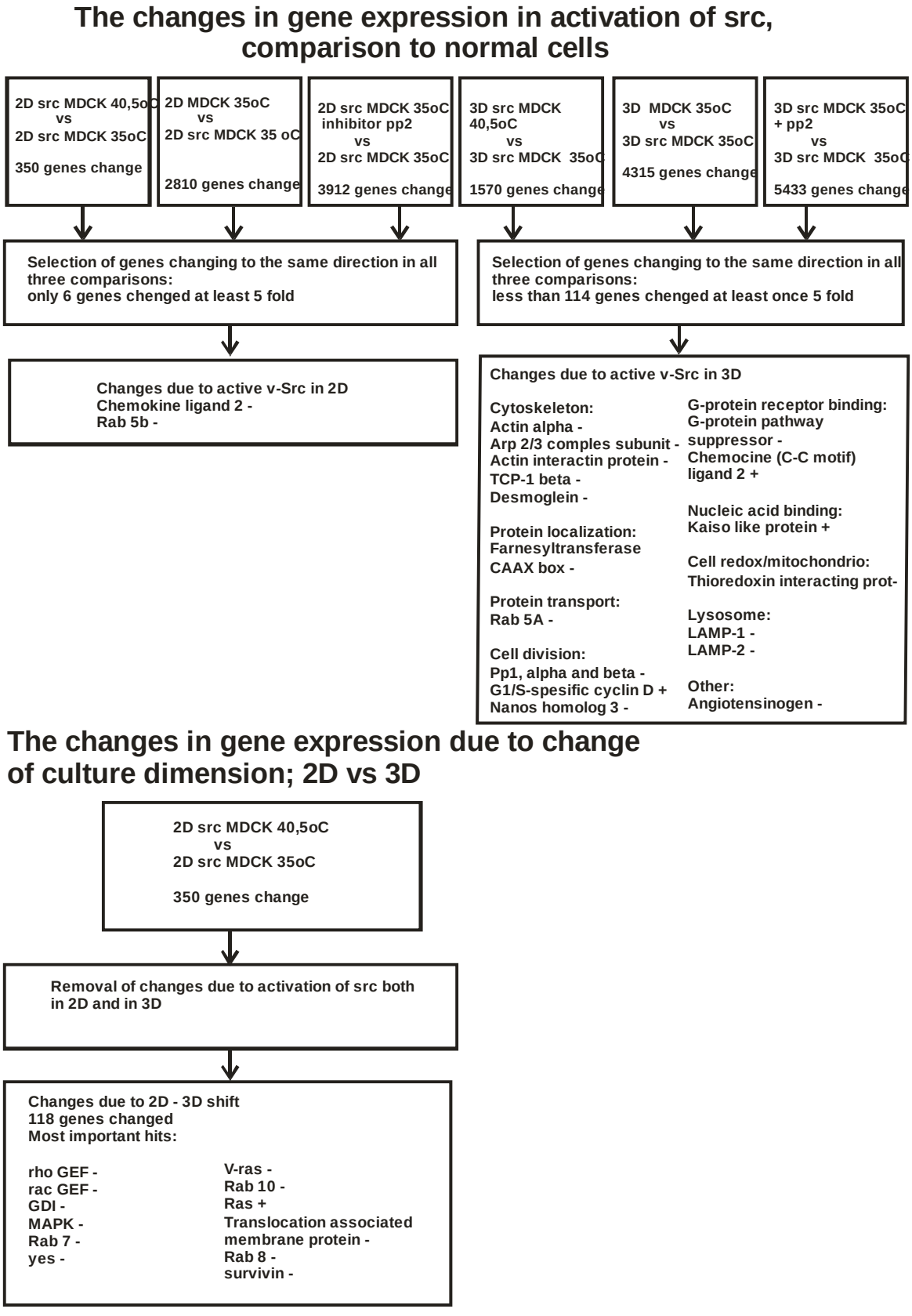


Figure 18 *Results of comparison of gene expression profiles of normal MDCK and ts-src MDCK cells in activation of src in 2D and in 3D and the results from 2D – 3D shift.*

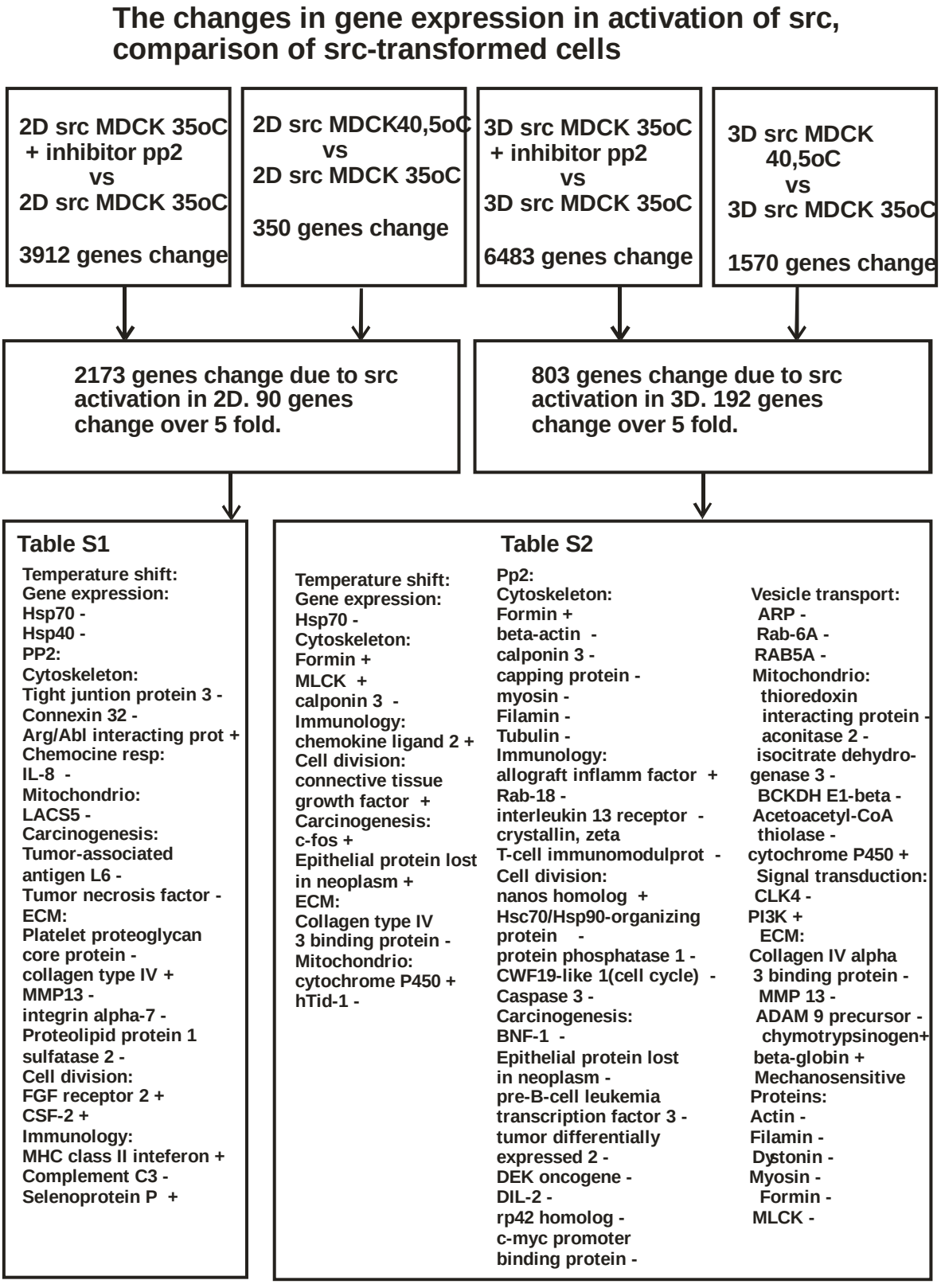


Figure 19 *Results of comparison of gene expression profiles of ts-src MDCK cells in activation of src in 2D and in 3D.*

5.3.2 Regulation of src-induced changes in genes involved in apoptosis in 2D and in 3D (II-III)

Morphological and immunofluorescence studies described above strongly suggested that apoptosis played an important role in the formation of the central lumen during the orderly establishment of a cyst-like architecture when ts-src MDCK cells were grown in 3D culture at non-permissive temperature. Conversely, mechanisms counteracting or offsetting the effects of apoptosis seemed to operate in src-transformed cells since no lumen formation was seen. Instead there was a phenomenon of filling of the lumen with cells of mesenchymal phenotype.

In order to explore the molecular basis of the role of apoptosis in lumen formation and in "filling of the lumen" phenomenon, we especially scrutinized the changes in the expression of apoptosis-related genes as detected by gene arrays. In this regard, we became especially interested in survivin, a well-known anti-apoptotic protein (Altieri, 2006).

Gene expression analysis showed that survivin was strongly expressed in both untransformed and ts-src transformed cells in 2D. This is consistent with the observation that no apoptosis was seen in 2D in either normal or transformed MDCK cells. In 3D, on the other hand, survivin was not expressed in nontransformed cells and ts-src MDCK cells at non-permissive temperature. It was, however, expressed in ts-src-MDCK cells at permissive temperature. Again, this is consistent with the observations that apoptotic cells were seen in MDCK and ts-src MDCK cells at non-permissive temperature in 3D, and that only a few apoptotic cells were seen in ts-src MDCK cells at permissive temperature. This suggests that src-induced expression of antiapoptotic protein survivin could be a mechanism by which the central cells in 3D cell clusters avoid apoptosis and thus, contribute to the phenomenon "filling of the lumen".

5.3.3 Verification of the altered expression levels of the genes associated with apoptosis by RT-PCR and western-blotting (II-III)

In order to verify the results of gene expression studies concerning the expression of survivin and its regulator PTEN, we also carried out RT-PCR and immunodetection studies. For that purpose MDCK and ts-src MDCK cells were cultured under following conditions:

1. MDCK cells in 2D and in 3D Matrigel at permissive temperature (35 ^{o}C) (II-III)
2. Ts-src MDCK cells at permissive temperature (35 ^{o}C) in 2D and in 3D Matrigel (II-III)
3. Ts-src MDCK cells at non-permissive temperature (40.5 ^{o}C) in 2D and in 3D Matrigel (III)
4. Ts-src MDCK cells at permissive temperature (35 ^{o}C) in 2D and in 3D Matrigel with pp2 (III)

RNA was extracted from the cultures and the appropriate primers for survivin and its regulator PTEN were used for RT-PCR.

The RT-PCR-studies showed that, in 2D, the expression level of survivin was high in both MDCK and ts-src MDCK cells and that it was not dependent on whether src was active or not. A dramatically lowered level of survivin was seen in MDCK and ts-src MDCK cells grown at non-permissive temperatures in 3D. In 3D, the level of survivin remained high in ts-src MDCK cells grown at permissive temperature with or without pp2 when the 3D cultures were compared.

In western blotting, a distinct band corresponding to survivin was seen in lysates of MDCK cells grown in 2D and of ts-src transformed cells grown in 2D. No expression was seen in MDCK cells or ts-src MDCK cells at non-permissive temperature in 3D. On the other hand distinct bands were seen in ts-src MDCK cells at permissive temperature with or without pp2.

The expression of survivin is regulated by a network of proteins. One of the most important of them is PTEN, a tumor suppressor that acts by silencing expression of survivin (Altieri, 2006; Guha et al., 2009). Therefore, we next wanted to compare the expression of PTEN relative to that of survivin in ts-src MDCK cells in 2D and in 3D both by using RT-PCR and western blotting.

MDCK and ts-src MDCK cells at permissive temperature showed a higher level of expression of PTEN in 2D than in 3D as judged by RT-PCR and western blotting when compared with ts-src MDCK cells at permissive temperature.

The expression of PTEN was high in MDCK and ts-src MDCK cells at non-permissive temperatures in 2D. The expression was low in ts-src MDCK cells at permissive temperature with pp2 in 2D and was very low in ts-src MDCK cells at permissive temperature in 2D.

In 3D, in MDCK cells and in ts-src MDCK cells at non-permissive temperature, the expression of PTEN was lower than in the same cells in 2D. Surprisingly, in ts-src MDCK cells at permissive temperature with or without pp2 in 3D, there was more PTEN detectable than in MDCK cells in 3D.

Summary of the results of RT-PCR and western blotting is shown in Figure 20.

Protein \ Cell type	MDCK		Ts src MDCK 35 oC		Ts src MDCK 35 oC + pp2		Ts src MDCK 40,5 oC	
	2D	3D	2D	3D	2D	3D	2D	3D
Survivin	++	+/-	++	+++	++	+++	++	+/-
PTEN	++	+	+/-	++	+	++	++	+

Figure 20 *Summary of expression of survivin and PTEN in various cell culture conditions by western blotting and RT-PCR.*

As a conclusion, it is obvious that in MDCK and ts-src MDCK cells at non-permissive temperature, i.e. in cells with polarized phenotype and lumen formation in 3D, the expression of PTEN is inversely correlated with the expression of survivin. In transformed cells, in which the polarization is deficient and the lumen formation is disturbed, the expression of survivin is not affected even though the same cells showed elevated level of PTEN. The results suggest that in src-transformed cells the function of PTEN on the regulation on the expression of survivin may be altered or the activation of other regulatory elements e.g. activation of the PI3K pathway be enhanced by src leading to elevated levels of survivin. The results from microarrays showed increased expression of PI3K among activation of src in 3D matrices (III).

Overall, the results from RT-PRC and western-blotting results are in line with the gene expression data and support the hypothesis that the failed lumen formation (or filling of the lumen) in ts-src MDCK cell in 3D is due to enhanced expression of survivin, either by direct gene regulation or indirectly via up- or downregulation of its regulators PTEN and/or components of the PI3K-pathway (II-III).

5.3.4 Localization of survivin in MDCK and in ts-src MDCK cells in 3D by immunofluorescent techniques (III)

Proteins may have quite different and even antagonistic functions depending on their subcellular localization. Survivin, for instance, has a dual role in inhibiting apoptosis and regulating proliferation of cells. It has been suggested that the cytoplasmic localization of survivin is linked to its IAP function, whereas its nuclear localization is a prerequisite for its proliferatory function (Angnell, 2008; Altieri, 2006).

Next we investigated the localization of survivin in MDCK and ts-src MDCK cells in 3D. For that purpose the cells were cultured in Matrigel as described before, immunostained with anti-survivin antibodies and observed under a confocal microscope.

In MDCK and ts-src MDCK cells at non-permissive temperature, survivin was seen to be associated with the mitotic spindle of dividing cells. On the other hand, there was also more diffuse cytoplasmic staining in ts-src MDCK cells. At permissive temperature, survivin was mainly localized in the cytoplasm, though it was associated also to the mitotic spindle of the dividing cells. In ts-src MDCK cells at permissive temperature with pp2 most of the staining was nuclear, although some survivin localized to the cytoplasm was also seen.

As a conclusion, in polarized cells survivin was seen only in association with the mitotic spindle and that the activation of src in MDCK cells is associated with a redistribution of survivin from nucleus to the cytoplasm This suggests that its role in apoptosis, as was suggested by experiments described above, is not only due to altered expression levels but also due to altered distribution between cytoplasm and nucleus.

5.3.5 Verification of the genes altered in association with cadherin recycling and endocytosis machinery by RT-PCR and western-blotting of cadherin (II)

Gene expression array analysis showed upregulation of expression of several vesicle transport proteins upon shift from 2D to 3D. Next we wanted to verify these changes by looking at the expression levels of the corresponding mRNAs and proteins by using RT-PCR and western blotting, respectively.

As judged by RT-PCR; a shift of MDCK cells from 2D to 3D environment brought about from 3 to 7 – fold increases in Rab5, 3-fold decrease in Rab7 and 5-fold decrease in Rab8. A comparison between ts-src MDCK cells at permissive temperature in 2D and 3D showed an increase in the expression levels of Rab5, Rab7 and Rab8. Overall, the expression of Rab5, Rab7 and Rab 8 was altered significantly upon changing the culture conditions from 2D to 3D. On the other hand src-transformation was associated with more distinct changes in Rab expression in 3D cultures than in 2D.

Secondly, we wanted to investigate whether src activation affected the expression of E-cadherin or induced expression of novel cadherins. This was partially motivated by the findings of the gene array analysis that, surprisingly, showed no major changes in the expression levels of cadherins upon src-transformation. On the other hand, it is known that, in EMT, epithelial cadherins are sometimes replaced by mesenchymal cadherins (Thiery, 2002).

In order to analyze the expression of cadherins, we used western blotting. MDCK cells were grown in 2D and in 3D Matrigel and ts-src MDCK cells were grown in 2D and 3D Matrigel at permissive temperature. For western blotting, antibodies to cytoplasmic tail of E-cadherin (rr1), pan-cadherin antibody and antibody to E-cadherin were used.

No major change in the expression levels of E-cadherin was seen in ts-src MDCK cells upon transformation in 3D as judged by western blotting. Neither was there any indication of an expression of novel cadherins.

The results above are in line with the results from gene expression analysis, that there were no major changes in the expression of cadherins.

Overall, the results suggest that, due to transcription level changes of genes regulating vesicular transport, endocytosis and protein transport are altered upon src-transformation. In addition to that, also changes in the actin cytoskeleton and deranged polarity impair the structure and function of the protein transportation machinery. The results also suggest that differences observed in the morphology of the cyst formation in 3D or the monolayer in 2D could be due to altered transportation and localization of E-cadherin in normal and transformed cells (II).

5.3.6 Src activity (II-III)

Next we wanted to verify that the temperature shift from non-permissive to permissive is associated with the anticipated changes in src-activity in ts-src MDCK cells.

In order to measure the src activity, we used Western blotting and antibodies against total src, active src and viral (avian) src. Antibody against activated Src (anti-src,

activated, pY416), antibody against Src (36D10) for total cellular Src; anti-avian Src, clone EC10 antibody for avian src were used for the study.

MDCK cells were grown in 2D and in 3D Matrigel. Ts-src MDCK cells were grown in 2D and in 3D Matrigel at non-permissive and permissive temperatures with or without pp2, and the activity of src was measured.

In 2D, the MDCK cells showed, in western blotting, positive signal only with the antibody to total c-Src. In the ts-Src MDCK cells grown both at 40.5 ^{o}C and 35 ^{o}C temperature, both avian Src and total c-Src were detected. Moreover, the signal for activated pY416 Src was very strong in specimens of cells grown at 35 ^{o}C and very faint in cells grown at 40.5 ^{o}C. Hence, v-Src was expressed both at non-permissive and permissive temperature, but activated only at permissive temperature of 35 ^{o}C. The src activity was identical in 3D when compared to 2D. The activity of src in various conditions is shown in Figure 21.

As a conclusion, all of the cell lines express normal c-src. Avian src was detected in all of the ts-src MDCK cells at both temperatures. Activated src was seen in ts-src MDCK cells at permissive temperature, even with the src-kinase inhibitor pp2 in the growth medium.

Activity of src in various conditions

antibody / Cell line	36D10 (total src)		pY416(active src)		EC10 (avian src)	
	2D	3D	2D	3D	2D	3D
MDCK	+	+	-	-	-	-
Ts src MDCK 35oC	+	+	+	+	+	+
Ts src MDCK 35oC+ pp2	+	+	+	+	+	+
Ts src MDCK 40,5oC	+	+	+/-	+/-	+	+

Figure 21 *Summary of src activity in 2D and in 3D.*

6 Discussion

6.1 2D and 3D cultures

Credit for the progress made in understanding the cell biology of normal and malignant cells is mainly due to the study of cells in 2D cultures. They have been widely used as standard models from which the knowledge has been applied to *in vivo* systems. 2D cultures are commonly used, even though it is recognized that they poorly mimic the conditions prevailing in living tissues (Cukierman et al., 2001; Lee et al., 2008). In fact, only recently more attention has been paid to the choice of culture method and its effect on the data, and how it may distort the conclusions extrapolated from cell culture studies to *in vivo* conditions (Yamada and Cukierman, 2007).

In 2D cultures, epithelial cells form a monolayer. In the monolayer, the cells attach to the cell culture substratum via their cell-matrix contacts located on the basal surface and to each other via specific cell-cell attachment sites and structures located at the lateral walls (Rodriguez-Boulan and Nelson 1989). Even under 2D conditions, epithelial cells are able to polarize with requisite cell-cell junctions along lateral walls and the apical side facing the cell culture medium (Tanos and Rodriguez-Boulan, 2008). However, these nearly isotropic 2D models are poor representations of the tissue structures *in vivo*, because they do not allow formation of the tubular structures native to many types of epithelial tissues (Cukierman et al., 2001; Pankov et al., 2005). 2D conditions also give rise to tissue culture artifacts, e.g. focal adhesions. They have been suggested to be merely cell culture artifacts because they do not exist *in vivo* (Seeberger et al., 2009). Currently, increasing attention has been paid to the experimental restrictions accrued by culturing cells in 2D (Yamada and Cukierman, 2007).

The 3D cultures have gained more interest as a link between 2D cultures and *in vivo* models. In fact, recent developments in cell culture technology have led to the establishment of several types of 3D culture systems (Griffith and Swartz, 2006). Unlike 2D cultures, in 3D cultures the interactions between cells are three-dimensional, and cells can differentiate and coevolve into 3-dimensional architectural structures, which at least mimic the native *in vivo* structures organizationally and functionally (Yamada and Cukierman, 2007).

Despite the apparent deficiencies of 2D culture models, there are only few reports describing how the change of cell culture conditions from 2D to 3D affects gene expression (Sung et al., 2005). It would be logical to expect that, when shifting from isotropic conditions to more complex environments, not only does the spatial arrangement of cells undergo changes, but that changes take place also at the functional and gene expressional levels.

One of the aims of this work was to improve 3D cell culture methods by using MDCK cells, one of the most widely used epithelial cell lines, as a model. Another aim was to analyze the malignant transformation of epithelial cells in 2D and in 3D by using a unique MDCK cell line that is temperature-sensitive to src-oncogene-induced transformation in a reversible manner.

6.2 Cell growth in 3D, the effect of cell type and culture conditions

In 3D cultures, several variables have to be taken into account when considering the generalizability of the results of an experiment: 1) cell type, 2) composition of the matrix and 3) density of the matrix.

First, the cell type is known to affect the outcome. Cells 'remember' their origins and follow their pre-programmed plan at least to make a caricature of their native tissue, even when grown in cell culture. E.g. cells from epithelial tissues form tubular or spherical structures also *in vitro* in 3D (Cukierman et al., 2002).

The cells used for cell cultures can be harvested from tissues (primary cultures), or already established cell lines can be used. There are thousands of distinct cell lines that can be used for modeling different tissues. The disadvantage of using immortalized cell lines is that they are always somewhat altered to adjust better to the cell culture conditions (Friedl et al., 2004). Even fibroblasts form 3D structures in gels, whereas in 2D culture conditions they remain flat (Yamada and Cukierman, 2007). Transformed cell lines or primary cell cultures from various malignancies can be used as models of different tumors (Knuechel and Masters, 1999).

Second, the composition of the matrix has a crucial effect on the behavior of the cells. Cells bind via integrins to their surrounding matrix (Burridge et al., 1988). Different cell types have different combinations of integrins, and therefore require different scaffolding matrix proteins to bind to in order to function normally (Lubarsky and Krasnow, 2003; Kleinman and Martin, 2005). Thus, for an optimal outcome, it is important to select an appropriately matched 3D *in vitro* matrix, e.g. kidney epithelium needs a different composition of proteins to bind to than do osteoclasts or neuronal cells (Yamada and Cukierman 2007).

Presently, there are commercial gels available to meet the needs of specific cell types. One of the most widely used gels is Matrigel, made of an extract from Engelbreth-Holm-Swarm (EHS) mouse sarcoma tumor. Its biochemical composition resembles the complex extracellular environment found in many tissues, and it is widely used as a substratum for culturing epithelial cells (Kleinman and Martin, 2005).

Third, the density of the matrix has an impact on the cellular phenotype. Cells adjust their internal tension via integrins to match the tension of the surrounding matrix (Mammoth and Finberg, 2009). Internal tension, on the other hand, has an impact on many intracytoplasmic signaling pathways and gene programs for differentiation (Ingberg, 2006; Yamada and Cukierman, 2007). Different tissues have typical densities that differentially affect the phenotypes of the cells. The same extracellular matrix protein may have different effects on cell behavior in different concentrations. With no matrix contact, e.g. in suspensions, the cells have no possibilities to bind. This leads to rounding of the cells and promotes apoptosis (anoikis) (Discher et al., 2005). Each cell type has an optimal matrix density in which to grow (Yamada and Cukierman, 2007). As a rule, a denser matrix favors a motile and more mesenchymal phenotype, whereas a softer matrix promotes polarization of epithelial cells (Paszek et al., 2005; Butcher et al., 2009).

6.3 Studying cancer in 3D cell cultures

Various approaches have been used in attempts to model the growth of cancer *ex vivo*. Such approaches are necessary in order to analyze and interfere with cancer growth in ways not applicable in *in vivo* manipulation and experimentation. The most widely used techniques to study cancer *in vitro* are primary cell cultures from tumors, established cell lines in cell culture models, and explants from tissues (Knuechel and Masters, 1999). From early on, cell culture studies have proved to be valuable in providing information, for instance, on the growth rate or mobility of malignant cells. Later on, they were instrumental in revealing the role of other characteristics or hallmarks of cancer (Hanahan and Weinberg, 2000). The 3D culture models are instrumental in studying anchorage-independent growth, the capacity of the cells to invade the surrounding matrix, proteolysis of the matrix, or deficient differentiation, and evasion of apoptosis (Yamada and Cukierman, 2007).

More recently, cancer biology has advanced from the characterization of the cancer cell structure and behavior to uncovering the genetic and molecular alterations that presumably underlie the malignant phenotype. The simplified 2D culture models have not, however, fulfilled their role to recapitulate the *in vivo* conditions faithfully. The recent data on the differences of the cell culture models suggest that a change from 2D to 3D culture conditions affects not only polarization, but also alters signaling pathways and gene expression (Yamada et al., 2007; Yamada and Cukierman, 2007).

The new data suggest that cell culture conditions and the degree of dimensionality in cell culture have a much greater role in altering not only cell morphology, but also cell signaling and gene expression as well. Therefore, it seems that all the data gathered from studies in 2D, for instance on the effect of single oncogenes on the growth of cancer cells, may have to be re-evaluated in 3D cancer models to gain more biologically relevant information. The re-examination of old oncogenes or cancer models in 3D cultures may bring better insight to the mechanisms behind cancer (Yamada et al., 2007; Yamada and Cukierman, 2007). 3D cell cultures have therefore proved to be indispensable to modern cancer biology due to their more authentic nature as compared to 2D models, and also due to their accessibility to molecular manipulation to the same degree as conventional 2D models (Yamada et al., 2007; Sakamoto et al., 2001).

6.3.1 The ts-src MDCK cell line – a model of epithelial differentiation and carcinogenesis

It is important that the *in vitro* models used in molecular studies recapitulate the *in vivo* situation as closely as possible. One of the aims of our study was to create novel methods of culturing cells in 3D conditions so that the 3D structures remain as intact as possible during immunostaining and imaging of the samples. In this study, we have used the ts-src MDCK cell line as a model of epithelial cell polarization and its disturbances in cancer.

The ts v-src MDCK cell line was created to study the role of E-cadherin and β-catenin in cell-cell adhesion (Behrens et al., 1993). The cell line was created by transfecting

MDCK I cells with a gene encoding a temperature-sensitive variant of v-src protein. The gene carries a mutation that makes its protein product temperature-sensitive. At 35 °C, the protein encoded by the transfected gene is active and the host cells are transformed. At 41 °C, the protein is denatured and, thus, inactive and the cells grow normally, displaying normal epithelial phenotypic features (Wyke and Lineal, 1973). The process is reversible; the cells can be quickly and repeatedly switched from one state to the other simply by altering the temperature. The transfected cells were distinguished by their morphology and colony-forming ability (Behrens et al., 1993).

Src is the oldest known oncogene, and it has been found in many cancers (Collett and Erikson, 1978). The src oncogene is asociated with cell survival and metastasis in tumors (Sakamoto et al., 2001). In 2D cultures, src-induced transformation is accompanied by disruption of adherens junctions and a switch from a cuboidal to a more fibroblast-like morphology and motile behavior (Behrens et al., 1993). Activation of the src oncogene also leads to activation of wnt- and PI3K-signaling pathways and altered gene expression (Engelman et al., 2006; Logan and Nusse, 2004).

Due to the nature of the temperature-sensitive activation of src in ts-src MDCK-cells, the transformation process in the cell line is transient; the cells have a normal epithelial phenotype at non-permissive temperature and a mesenchymal phenotype at permissive temperature (Behrens et al., 1993; II, III). The inducible model is easy to use. Apart from the temperature shift, no additional ingredients need to be added to the culture to trigger the transformation. In spite of its validity as a model for physiological polarization, and its many apparent advantages, the cell line has not gained wider use in cancer biology. One of the reasons could be that the model has been criticized for being src-kinase-specific. On the other hand, concern has been expressed that the v-src expression is ‘leaky’, i.e. the v-src gene product would be active also at non-permissive temperatures and, therefore, would interfere with cell functions (Behrens et al., 1993).

Optimization of 3D culture conditions

Various methods have been used to emulate *in vivo* conditions in *in vitro* studies of epithelial cell polarization. The 3D *in vitro* equivalent of glands and tubular structures seen in epithelial tissues *in vivo* is a cyst, which can be described as a sphere of cellular mass with a central lumen or cavity surrounded by a layer of polarized cells (Nelson, 2003; Debnath and Brugge, 2003).

In order to obtain, in 3D culture, the best resemblance to native conditions in tissues, several factors have to be taken into account. First, the protein composition of the matrix affects the outcome. As a rule, epithelial cells thrive and gain greater similarity with the cells in tissues when grown in matrices composed of proteins of basal lamina than on matrices consisting of single components (Kleinman and Martin, 2005). Second, the density or stiffness of the matrix has an impact on cell behavior. Even the same protein at different relative concentrations promotes distinct phenotypes (Disher et al., 2005). Third, the volume or thickness of the gel-like matrix has an effect on the phenotype, due to the

degree of oxygenation and of the nutritional status of the cells (Keith and Simon, 2007; Levenberg, 2005).

In our study, we used several methods to culture cells in 3D. At the beginning of the present study, we had no standard protocols for epithelial cell cultures in 3D.

We started with Setup 1, in which the cells were cultured inside large volumes of matrix. The matrix provided the necessary mechanical support. However, there were problems; first, in the penetration of antibodies to the gel and, second, in preserving the original 3D structure throughout the immunostaining and visualization protocols, when protease treatment was used to improve the penetration of the antibodies. This was mainly due to the large volume of the gel and its viscosity in the cultures (I).

In order to overcome the problems in Setup 1, another arrangement, Setup 2, was used in the subsequent studies (II-III). In Setup 2, we modified the method created by Debnath et al. (2003) in which the cells are not embedded in matrix but, instead, grown on top of it. This allows only a part of the evolving sphere or cyst to be in contact with the matrix. Therefore, in order to ensure more uniform exposure to the matrix components, ECM proteins were added to the culture medium. Because there was less hindrance to the penetration of antibodies, this method enabled us to use easier and more accurate immunolabeling methods, and facilitated monitoring of the functional state of the cells.

In the third study, in Setup 3, the method was developed even further. Now the cells were embedded and grown within a gel that was thinner than in Setup 1. These conditions provided the cells with a 3D environment, the matrix proteins, and the mechanical support needed. The thinness of the gel, on the other hand, enabled unimpeded immunostaining and visualization of the specimens.

All the experimental arrangements provided a favorable 3D environment and matrix proteins needed for proper functioning. Setups 1 and 3 provided sufficient mechanical support needed for the spheres or cysts to form. Setups 2 and 3 allowed the use of more convenient and reproducible immunolabeling methods. As a conclusion, Setup 3 provided an optimal 3D environment for cell growth and for the immunolabeling of the 3D samples for microscopy.

Optimization of immunostaining and imaging techniques

Currently, confocal fluorescence microscopy represents a widely used and an advanced imaging technique for moderately thick specimens (Pampaloni et al., 2007). Thus, it is well suited for studies on rather voluminous aggregates formed of cells grown in 3D gels. The method has limitations, however. For example, the excitation light illuminates the entire depth of a sample during every step of focal recording. This causes photobleaching and has phototoxic effects at all planes. Another big problem is the limited penetration depth of the excitation beam, especially when lenses with a high numerical aperture are used (Verveer et al., 2007). Two or multiple photon microscopes allow deeper penetration to the sample, but the images are normally of lower resolution, and the bleaching of the focal plane is intensive (Zipfel et al., 2003). Other methods, such as optical coherence

tomography (OCT) and optical projection tomography (OPT) can be used for imaging larger and thicker samples (Huang et al., 1991; Sharpe et al., 2002).

The recent rapid development of computers and confocal microscopes enables faster scanning of multiple channels with higher resolution, and therefore helps to minimize the bleaching (Pampaloni et al., 2007). This became apparent during the course of our own studies. Bleaching was a problem in our early experiments in which we used equipment with a low scanning speed. Due to photobleaching, an attenuated signal or no signal at all was obtained from the more centrally located cell mass. Consequently, this lack of a signal from a marker protein was occasionally interpreted to mean that cells with particular properties were not present in that location. Such misinterpretation was avoided when a microscope with a faster scanning speed – and consequently less photobleaching – was used.

Cyst formation of MDCK cells in 3D as a model of epithelial differentiation

MDCK cells provide a classical model of epithelial cell polarization and differentiation. In 2D, they form a monolayer, but when grown in 3D in collagen I or Matrigel, they form spherical cysts, which are spherically arranged monolayers encapsulating a hollow cavity. From such studies, the basic difference in polarization between 2D and 3D has been explicated as follows: In 2D, the determinants of polarization are provided by the vectorial cues from the matrix which underlies the cells. In 3D, on the other hand, the process of establishing the spatial architecture cannot be based on such almost dichotomous cues, due to the isotropism of the growth medium. Polarization in 3D is thus based on an autonomous program initiated, but not guided, by the ECM (Pampaloni et al., 2007).

MDCK cells polarize also in suspension culture. However, in suspension they acquire inverted polarity, e.g. instead of the lumen, the apical surfaces are oriented outwards from the center and towards the surrounding growth medium (Wang et al., 1990). This difference is due to the differences in the direction of the secretion of the ECM proteins produced by the MDCK cells themselves. In 3D matrices, the proteins are secreted outside of the cell sphere, whereas in suspensions the ECM proteins are secreted inside the emerging lumen. Binding of the cells to the ECM proteins via integrins subsequently induces further polarization and guides the orientation of the polarity (Wang et al., 1990).

It is known from previous studies that normal MDCK cells have a spontaneous tendency to polarize (Wang et al., 1990). In 3D matrices, they form a cyst with a peripheral rim of polarized cells with a lumen in the center, and the apical surfaces of the peripheral cells face the lumen.

There are basically two mechanisms by which the lumen formation can be accomplished. They are known as cavitation and hollowing (Martin-Belmonte et al., 2008).

Cavitation occurs when a group of cells proliferate in an adhesive manner, which is initially only moderately polarized. However, the selective apoptosis of cells that are not in contact with the ECM gives rise to an outer epithelial layer surrounding a now hollow lumen (Martin-Belmonte et al., 2008, Bryant and Mostov, 2008).

During hollowing, intracellular vesicles are formed at the cell surface at a coordinated point between two closely apposed cells, creating a luminal space between polarizing groups of cells. These vesicles contain fluid and apical proteins, which are destined for delivery to the lumen. The surrounding cells now exhibit apico-basal polarity and are orientated around a lumen. The entire tissue subsequently expands in a highly polarized manner.

From previous studies it is known that MDCK cells can utilize both cavitation and hollowing to form the lumen. Choosing one or the other program in 3D depends, e.g. on the nature of the matrix and the density of the cells in the gel (Martin-Belmonte et al., 2008).

In the present study, we showed that ts-src MDCK cells at non-permissive temperature in Matrigel form a cyst with a central lumen similar to the one seen in normal MDCK cells. The lumen was formed via apoptosis of the cells without matrix contact, as judged by immunofluorescent techniques and based on measurements of mitochondrial metabolism. The peripheral cells of the cysts formed by ts-src MDCK cells at non-permissive temperature displayed characteristics of polarity similar to those of normal MDCK cells: adherens junctions binding the neighboring cells together laterally, and tight junctions formed apically. Thus, for the purposes of this study, ts-src MDCK cells can be considered to be a valid model for MDCK cell differentiation in studies focusing on transformation-associated derangements of polarization, and lumen formation induced by a temperature shift to manifest the transforming capacity of the transfected src gene.

Defective cyst formation and polarization of MDCK cells in 3D as a model of carcinogenesis

Upon transformation, epithelial cells, such as MDCK cells, lose their polarized organization and become flattened. This is accompanied by weakening of cell-cell junctions (Schoenenberger et al., 1991; Behrens et al., 1993; Takeda and Tsukita, 1991). Ts-src MDCK cells, when grown at permissive temperature, lose their polarized, strictly epithelial phenotype and acquire a fibroblast-like morphology as a sign of transformation (Behrens et al., 1993; Sormunen et al., 1994). Similarly, MDCK cells transformed with an active Ras oncogene exhibit a mesenchymal phenotype (Schoenenberger et al., 1991).

Several studies have described cyst formation in transformed epithelial cells grown in 3D matrices. Breast carcinoma cell lines are commonly used to study cancer, e.g. MCF-10A forms dome-like structures and a cavity in 3D, but the Ras-transformed MCF-10A cell line shows no signs of polarization nor lumen formation. Instead, the central area in the emerging spheroids remains filled with vital cells (Bissell et al., 2002).

Defective cyst formation is a common finding in malignant epithelial cells in 3D, so that instead of a lumen forming in the center of the cellular sphere, cells continue to fill the central space. The failure to form a lumen in 3D resembles the situation in early cancers. This phenomenon is considered to be biologically equivalent to the glandular structures that are filled with vital cells *in vivo* (Bissell et al., 2002; Debnath et al., 2003). It has been called ‘filling of the lumen’, which is seen in many early epithelial cancers (Hanahan and

Weinberg, 2000). Typically, the cells in the central area are not, however, quite similar to the polarized cells in the peripheral rim but, instead, have a more mesenchymal or fibroblastic phenotype.

Due to the importance of the 'filling of the lumen' phenomenon in epithelial carcinogenesis, we decided to look more closely at the determinants of defective lumen formation in ts-src MDCK cells at permissive temperature. These studies were motivated especially by observations that point to the importance of apoptosis in physiological lumen formation by cavitation (Martin-Belmonte et al., 2008). Conversely, failure of or deficient lumen formation could be due to defective or suppressed apoptosis, or its absence. Indeed, for instance in cell line MCF10-A, deficient apoptosis has been shown to play an important role in the lack of a central lumen in these cell clusters (Bissell et al., 2002; Debnath et al., 2002; Debnath et al., 2003; Debnath and Brugge, 2005; Danes et al., 2008).

The culture conditions are of central importance. We found that the effects of transformation, upon src activation, on cyst and lumen formation are very different in different matrices. In a soft matrix, which is rich in ECM proteins (Matrigel), the cells formed a sphere with two cell populations. The cells in contact with the matrix had a more epithelial phenotype with adherens junctions along the lateral cell walls. The cells in the center without matrix contact remained viable, but had a mesenchymal phenotype. Thus, vital cells with a mesenchymal phenotype filled the space which in nontransformed cells was destined to be the lumen of the cyst.

In a stiffer matrix (collagen I), there was no cyst formation. Instead, the cells formed a non-oriented sphere with several protrusions. The cells showed no signs of polarization, and no central lumen was formed. When the ECM protein laminin was added to the matrix made of collagen I, a loss of protrusions was seen, suggesting a change in the motility of the cells. Concomitantly, the cell-cell junctions became more distinct, as judged by immunofluorescence microscopy, also attesting to at least partial restoration of the non-motile epithelial phenotype. However, even when laminin was added, no signs of lumen formation were seen. Thus, Matrigel partially either restores the epithelial phenotype deranged by src-transformation or, alternatively, prevents any src-transformation associated events. This is not enough to overcome the failed lumen formation, however.

Immunofluorescence analysis showed distinct differences in the organization of cell-cell junctions between cells grown in collagen I and in Matrigel. Especially the occurrence of ZO-1 in the cells grown in Matrigel, but not in the cells grown in collagen I. suggests that the more polarized phenotype in the former is at least partly due to the less disturbed tight junctions and possibly also other cell-cell junctions in the Matrigel-grown cells. Also the distribution of fodrin was different: in cells grown in collagen I it appeared in the cytoplasm, and in Matrigel-grown cells along the cell walls. From previous studies it is known that membrane association of fodrin is a sign of polarized phenotype in epithelial cells (Sormunen et al., 1994). Thus, based on these markers, src-transformed cells in Matrigel seem to acquire or maintain a distinctly more polarized phenotype than in the other, mostly collagen I-based matrices tested in this study. Regardless of the molecular mechanisms of the counteracting effects of Matrigel on src-transformation, it seems

probable that at least ZO-1 expression is regulated at a transcriptional level, while the redistribution of fodrin and cadherin is a post-translational event.

Experiments with collagen I matrices supplemented with various basement membrane proteins, and those supplemented with the conditioned medium from the cultures in Matrigel, showed that the preservation of polarized phenotype despite src activation was not due to any of the tested matrix components, but rather to the soluble factor. Further experiments revealed TGFβ1 as the most probable candidate to mediate this effect. This view was strengthened by the finding that the polarized phenotype could be reinstated upon treating the transformed MDCK cells with TGFβ1, when they were grown in growth factor-depleted Matrigel.

Our findings on TGFβ1 as a polarization-inducing or -maintaining agent are supported by studies showing that TGF isoforms TGFβ1, TGFβ2 and TGFβ3 inhibit proliferation of epithelial cells and induce matrix protein synthesis and integrin expression (Piek et al., 1999). Further biological effects of TGFβ, which could underlie the effect seen in this study, are its inhibitory impact on the synthesis of proteases that degrade matrix proteins, and its enhancement of the synthesis of the inhibitors of various proteases (Sastry and Horwits, 1996; Wright and Huang, 1996). Most interestingly, TGFβ has been shown to induce degradation of active v-src kinase in fibroblasts, most likely by controlling the activity of several proteases (Fukuda et al., 1998 a, b; Kuzuya and Kinsella, 1994). Furthermore, it has been shown that epithelial cells can be induced to differentiate with the combined effect of scatter factor, EGF, TGFα, bFGF and IGF-1 (Sakurai and Nigam, 1997).

Deficient apoptosis in transformed MDCK cells in 3D cultures

The ts-src-transformed cells at non-permissive temperature in Matrigel formed a cyst with a central lumen. The lumen was formed by cells without matrix contact, trapped inside the lumen; the cells underwent apoptosis based on their expression of apoptotic markers. This event was similar to the lumen formation in normal MDCK cells (Martin-Belmonte et al., 2008). Activation of src led to a different phenotype. When ts-src MDCK cells were grown at permissive temperature in Matrigel, instead of a lumen, the central core area was filled with vital cells with no sign of ongoing apoptosis. This was more clearly seen in the cells grown in collagen I matrix; there was no lumen formation and the centrally located cells showed no signs of apoptosis. Thus, in line with other studies (Hanahan and Weinberg, 2000, Bissell et al., 2002; Debnath et al., 2003 and 2003), cell growth that is deranged, defective or lacking in apoptosis seems to be a striking feature of missing lumen formation when transformed MDCK cells are grown in 3D culture.

6.3.2 Changes in gene expression related to transformation in ts v-src-transformed MDCK cells in 2D and in 3D

There are only a few comparisons of the gene expression profiles in normal and src-transformed cells. However, there are some reports of the overexpression of DNA methyl transferase 1 and down-regulation of several tumor suppressor genes in fibroblasts upon src-transformation (Sung et al., 2005). On the other hand, in chicken embryo fibroblasts transfected with Rous sarcoma virus (RSV), vasoactive intestinal polypeptide and MAP were upregulated, and kinase phosphatase 2 and follistatin were down-regulated upon transformation (Masker et al., 2007).

In order to find the genes whose regulation was affected by src-transformation of MDCK cells in 2D, filtering was performed as follows: Only those genes were selected for further analysis that were consistently either up-regulated or down-regulated in all the comparative settings applied, i.e. with 1) MDCK cells, 2) v-src-transformed MDCK cells at non-permissive temperature, and 3) v-src transformed cells in the presence of pp2.

Due to the loss of cell polarity observed in src-transformed cells in 2D, we anticipated that expression of the genes involved in cell polarization would be affected. Surprisingly, however, no changes were seen in the expression levels of genes encoding junctional proteins, such as cadherins or catenins. It is thus probable that the changes seen in cellular phenotype are due to post-translational modifications that lead to altered protein-protein interactions. Phosphorylation of the junctional proteins probably plays a decisive role, as shown earlier (Palovuori et al., 2003).

A similar analysis was performed on ts-src MDCK cells in 3D in Matrigel. Mainly the genes involved in cell signaling, cell proliferation, energy metabolism and vesicle transport, were differentially regulated. The genes encoding cell-cell junctional proteins displayed no major changes. Interestingly, however, two genes, Nanos and Kaiso, which are closely linked to the regulation of cell-cell junction proteins, were upregulated upon src-activation.

Both Kaiso and Nanos are associated with localization and expression of E-cadherin and p120 catenin. Kaiso is a repressor of transcription and is linked to the β-catenin/TCF/LEF pathway. It shuttles between the cytoplasm and the nucleus, where one of its functions is to repress sequence-specific gene targets. Kaiso is also thought to have gene-regulating or cytoplasmic functions that may be subject to p120ctn modulation (van Roy and McCrea 2005).

Nanos is a p120 catenin-binding protein which, when overexpressed, brings about the release of junctional proteins into the cytoplasm, with a concomitant disintegration of junctional complexes (Strumane et al, 2006). Thus, up-regulation of Nanos and Kaiso by src could explain some of the effects of src activation on the junctional complexes, even if no major changes occur in the expression of the genes encoding junctional proteins.

Also the changes in the expression levels of genes encoding proteins involved in apoptosis (such as survivin and caspase 3), in cytoskeletal structures (such as formin, actin and filamin), in mitochondrial function and cell metabolism (such as thioredoxin-interacting protein and acetoacetyl-CoA-thiolase) are noteworthy, and in concordance with the phenotypic changes seen in MDCK cells upon transformation. Especially worth

mentioning are changes in the expression of some mechanosensitive proteins, such as actin, dystonin, formin and filamin. They may very well play an important role in the varying responses and sensitivities of normal and transformed MDCK cells when grown in matrices of varying densities. Mechanosensitive proteins are instrumental in mediating the density-related environmental cues to the internal signaling mechanisms of the cells (Inberg, 2006). Thus, their altered expression in response to changes in their external milieu may have a distinct effect on the cellular phenotype (Mammoto and Ingberg, 2009; Inberg, 2006).

Survivin was one of the overexpressed proteins in the src-transformed cells in 3D. The apparent significance and biological relevance of its enhanced expression derives, on the one hand, from the well-established function of survivin as an anti-apoptotic protein and, on the other hand, from the apparently failed or missing apoptosis in the center of the spheroids of the transformed MDCK cells. The expression of survivin is upregulated 24-fold in the course of the time span when the temperature is shifted from non-permissive to permissive. During this time, morphological alterations in the ts-src MDCK cells take place: from polarized cyst to the filling of the lumen phenotype. It is thus logical to propose that the failure of lumen formation is due to the anti-apoptotic effect of survivin on the central cells that otherwise would be destined to cell death. Support for this argument also comes from other studies which have shown that the level of survivin is very low in normal adult tissues, but clearly elevated in cancers (Altieri, 2008).

Apart from its anti-apoptotic effect, survivin might also affect derangements in cyst formation via its role in cell-cell adhesion. Earlier studies have demonstrated a marked down-regulation of survivin in cultured endothelial cells, upon cell adhesion and polarization and induction of contact inhibition of cell growth. On the other hand, expression of survivin is up-regulated in areas of growing vessels where VE-cadherin is partially dismantled (Iurlaro et al., 2004). Indeed, there seems to be an inverse correlation between expression of survivin and important cell-cell junction proteins. Similarly, in some epidermal and pancreatic cell lines and in ovarian tumors, E-cadherin and survivin expression are inversely correlated (Torres et al., 2008; Syed et al., 2008). Because survivin has a loosening effect on cell-cell junctions, its expression might also play a role in the altered motility of the cells.

There is still one aspect in the role of survivin in cell-cell adhesion that could be of importance in these putative survivin-mediated events in cyst formation. The expression of β-catenin and cadherins seems to suppress the expression of survivin in various cell types (Iurlaro et al., 2004; Torres et al., 2008; Syed et al., 2008). Thus, the enhanced expression of survivin in src-transformed cells in Matrigel could also be a consequence of the derangements in the junctional complex integrity that could, in turn, originate from other direct or indirect actions of src.

6.3.3 Changes in gene expression in relation to a switch in the dimensionality of growth of ts v-src MDCK cells

The phenotypic changes associated with the dimensionality of growth, from 2D to 3D, are well described and known to be associated with the polarization machinery. Also the most instrumental changes responsible for lumen formation in 3D are well known, involving cell-cell junctions, cytoskeleton components and apoptotic machinery. Indeed, at the biochemical level, changes in the expression, distribution and post-translational modification of cadherin and catenins and the GTPase proteins of the Rho-family have been found to be important (Drees et al., 2005; Yamada et al., 2005; Calpado and Macara 2007).

Nevertheless, little is known about the changes associated with the switch in growth dimensionality at the level of gene regulation. We used gene array technology to detect the changes in gene expression, between the 2D and 3D culture conditions We observed changes in the expression of over 6000 genes, when MDCK cells were cultured in 3D Matrigel instead of as monolayers. An unexpected observation was that there were no changes in the genes encoding cytoskeletal or cell-cell junction proteins. Neither were there changes in genes encoding proteins of apoptotic machinery, such as BIM and BAD, which previously have been associated with apoptotic lumen formation in epithelial cells (Dehan et al., 2009).

In the switch from 2D to 3D, the unaltered state of genes that are customarily associated with the polarization of epithelial cells suggests that only post-translational modifications are needed to build up a polarized phenotype in these cells. Thus, in the genetic sense, the genetic programs of cyst formation – and its associated polarization programs – seem to be launched in these cells already prior to the emergence of the conditions that make the execution of the program an option. Obviously, there could also be thus far undiscovered genes involved, or genes that were not represented in the arrays that we used.

Among the genes that were affected by the 2D-3D shift, there are some important GTPase proteins of the Rho family, which are important cytoskeletal regulators. Another example of the differences between 2D and 3D culture conditions is the observation concerning human epidermal growth factor receptors HER2 and HER3. In 2D culture conditions of cancer cells they form heterodimers, whereas in multicellular spheroids HER2 homodimers are formed, resulting in completely different downstream signaling cascades (Pickl and Ries 2006).

6.3.4 Mitochondrial dysfunction in src-transformed MDCK cells

In the present study, the metabolic state of mitochondria was monitored with a metabolic index, which gave the ratio between all mitochondria and those mitochondria with oxidative capacity (Buckman et al., 2001). It was used to assess the relative number of cells undergoing apoptosis.

Src-transformed cells displayed an elevated metabolic index as a sign of mitochondrial malfunction in 3D cell cultures when compared to normal MDCK cells. Ts-src MDCK cells at non-permissive temperature had the highest metabolic index. This coincided with the widespread apoptosis in the inner cell mass that was not in contact with the matrix. This result was obvious both in suspension cultures and in 3D. Thus, the occurrence of apoptosis was verified not only by immunohistochemical techniques (anti-caspase antibodies) but also by showing compromised mitochondrial function in the cells in which src is active.

Src-transformed cells in Matrigel at permissive temperature showed no apoptosis in spite of their mitochondrial malfunction. This ostensibly discrepant result could be due to the overexpression of survivin which, depending on the conditions, can also inhibit activation of caspases to their active form, especially when it is present in high concentrations. Survivin could thus inhibit apoptosis even in cells with malfunctioning mitochondria. Our results are in line with previous studies suggesting that ts-src MDCK cells, like other cancer cells, survive with survivin and enable filling of the luminal space (Holley and St Clair, 2009).

6.3.5 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition is a common phenomenon in epithelial cancers: the epithelial cells lose their characteristic features and acquire a mesenchymal morphology and become more motile (Hay, 1995).

The ts-src MDCK cells in 2D cultures at non-permissive temperature formed a polarized epithelium of cuboidal cells. Upon activation of src, the adherens junctions were disrupted, and morphologically the cells became long and more motile fibroblastic cells, both of these features being typical of EMT. Also in 3D, EMT was seen upon transformation. However, the morphology of the cell population was different, depending on the composition and density of the matrix.

Apart from the suppression of apoptosis, also EMT induced by src-transformation contributes to the filling of the luminal space and, in the case of collagen I matrix, to a spherical formation without orientation, and to invasion to the surrounding matrix. The findings suggest that deficient lumen formation and filling of the lumen are at least partly due to the enhanced mobility of the cells undergoing EMT. Our findings support the data from recent studies; i.e., the disruption of cell-cell adhesion enables the more motile phenotype seen in transformed tissues (Behrens et al., 1993; Kalluri and Weinberg, 2009).

One of the central features of EMT is the derangement of the structure and function of cell-cell junctions (Kalluri and Weinberg, 2009). In normal epithelial tissues, cell-cell junctions are continuously rearranged so that the epithelium is able to remodel in response to various stimuli (Fagotto and Gumbiner, 1996). The formation of cell-cell junctions is initiated by the neighboring cells coming in contact with each other. This is followed by accumulation of E-cadherin and ZO-1 at the lateral cell walls. E-cadherin is then released from ZO-1 to form adherens junctions (Rajasekaran et al., 1996). Cadherins then guide the

formation of the cell-cell junctions characteristic of the epithelial phenotype (Fagotto and Gumbiner, 1996).

In EMT, the cells typically replace the apical-basolateral polarity by antero-posterior polarity (Condeelis et al., 2005). This transformation is accompanied by the disruption of adherens junctions and internalization of E-cadherin, both of which are hallmarks of ongoing EMT and acquisition of a mesenchymal phenotype (Thiery, 2002). These events are regulated mainly by tyrosine phosphorylation of the components of the adherens junctions (Behrens et al, 1993; Palovuori et al., 2003; Alema and Salvatore, 2007; Miyashita and Ozawa, 2007). Indeed, in src-transformed cells, β-catenin and p120catenin become highly phosphorylated, leading to dislocation of the E-cadherin-β-catenin complex to the cytoplasm, and to weakening of the E-cadherin-mediated cell adhesion. Regarding the absence of any major changes in the genes encoding the adherens junction component, it is quite likely that the EMT seen in src-transformed MDCK cells in 3D in Matrigel is due to direct phosphorylation of the junctional proteins by src (Behrens et al., 1993; Palovuori et al., 2003; Alema and Salvatore, 2007; Miyashita and Ozawa, 2007).

It is well established that non-receptor tyrosine kinases, such as src, are capable of phosphorylating the cytoplasmic tail of E-cadherin (Fujita et al., 2002). This results in the internalization of E-cadherin. Such internalization of E-cadherin was seen in our study with GFP-tagged E-cadherin, lending further support to the argument that the EMT seen upon src-transformation is largely due to direct phosphorylation of junctional proteins (Behrens et al., 1993; Palovuori et al., 2003**).**

At permissive temperature, there was extensive disassembly of the E-cadherin-β-catenin complex, as indicated by the low colocalization coefficient. This suggests that at permissive temperature, cadherin is internalized via the clathrin-mediated pathway in which β-catenin is released from cadherin (Torres et al., 2007). The molecular basis of their differential distribution remains to be clarified. It may be partly explained by differential expression of several Rab-proteins associated with vesicle transport, as discussed below, or by the disintegrated cytoskeletal structures upon src-transformation.

The experiments with the pN1-ECad-EGFP-expressing ts-src MDCK cells in 3D also showed that, upon src transformation, the disruption of adherens junctions occurs rapidly. The weakening of cell adhesion was followed by increased mitotic activity, and the luminal space filled within a few days.

6.4 Role of survivin and PTEN in v-src transformation

Survivin is the smallest member of the IAP family of proteins. The level of survivin in differentiated cells and tissues is very low or not detectable at all (Li and Altieri, 1999; Altieri, 2006). On the other hand, survivin is expressed in most tumors (Fukuda and Pelus, 2006). Functionally, its expression is related to cell survival and inhibition of apoptosis (Altieri, 2006). Survivin localizes in the mitotic spindle and guides chromosomes to the polar ends of dividing cells (Jeyaprakash et al., 2007). It protects the cells from apoptosis by inhibiting the cleavage of caspases to their active form after mitochondrial damage.

Cytoplasmic and nuclear staining of survivin in cancers is an indication of a poor prognosis (Mehrotra et al., 2010).

In the present work, survivin in MDCK cells as well as in ts-src MDCK cells can be interpreted to have both an antiapoptotic and a proliferation-enhancing role. In 2D, the level of survivin was high in both non-transformed and transformed cells. This can be explained by the continuous division of the cells in 2D to the full capacity of the surface area of the cell culture dish. In 3D cultures in Matrigel, however, differences were seen in the expression of survivin between non-transformed and transformed cells. The survivin in the nontransformed MDCK cells localized in the nucleus, and was associated with the mitotic spindle. In the transformed MDCK cells at permissive temperature, on the other hand, cytoplasmic and nuclear staining was seen. Based on previous studies, it can be concluded that nuclear localization is indicative of proliferation, whereas cytoplasmic localization is related to the inhibition of apoptosis (Agnell 2008). All in all, our finding of the localization of survivin outside the mitotic spindle in the cytoplasm suggests that survivin also promotes proliferation in src-transformed MDCK cells. The decisive anti-apoptotic role of survivin in the failure of apoptosis in src-ts MDCK cells is supported by previous studies that have demonstrated the role of survivin in cell survival and filling of the lumen in other cell culture models (Altieri, 2006; Li and Altieri, 1999; Guha et al., 2009).

The importance of survivin as a mediator of some of the src-induced alterations in MDCK cells is based primarily on the observed expression levels of survivin in nontransformed and transformed MDCK cells. They can also be modified by other proteins that either suppress or enhance the expression of survivin. For instance, PTEN regulates the level of survivin via AKT. A high level of PTEN expression has a suppressive effect on the levels of survivin, but PTEN has also other functions. It acts as a polarity protein guiding PIP2 to the apical membranes. PIP2, in turn, brings about binding of annexin 2 and Cdc42 to the apical membranes. This subsequently induces the formation of the lumen (Martin-Belmonte et al, 2008; Engelman et al., 2006). A lowered level of PTEN could thus be expected to be associated with higher levels of survivin. However, in the present study no such clear-cut correlation was found, suggesting that more complex interactions and regulatory networks are involved.

PTEN can also be affected directly by active src due to the ability of src to reduce the stability of PTEN. This leads to a release of the AKT pathway from the suppressor effect of PTEN. Src also directly activates the PI3K pathway, and reverses the activity of PTEN, resulting in increased phosphorylation of AKT.

This process, in turn, leads to enhanced expression of survivin (Altieri 2006). Our gene array findings showed that the activation of src is associated with the upregulation of PI3K. Our findings are in line with previous data indicating that PTEN, upon src transformation, loses its regulatory function on survivin (Altieri, 2006; Li and Altieri, 1999; Lu et al., 2003). Figure 22 shows the pathways regulating the expression of survivin.

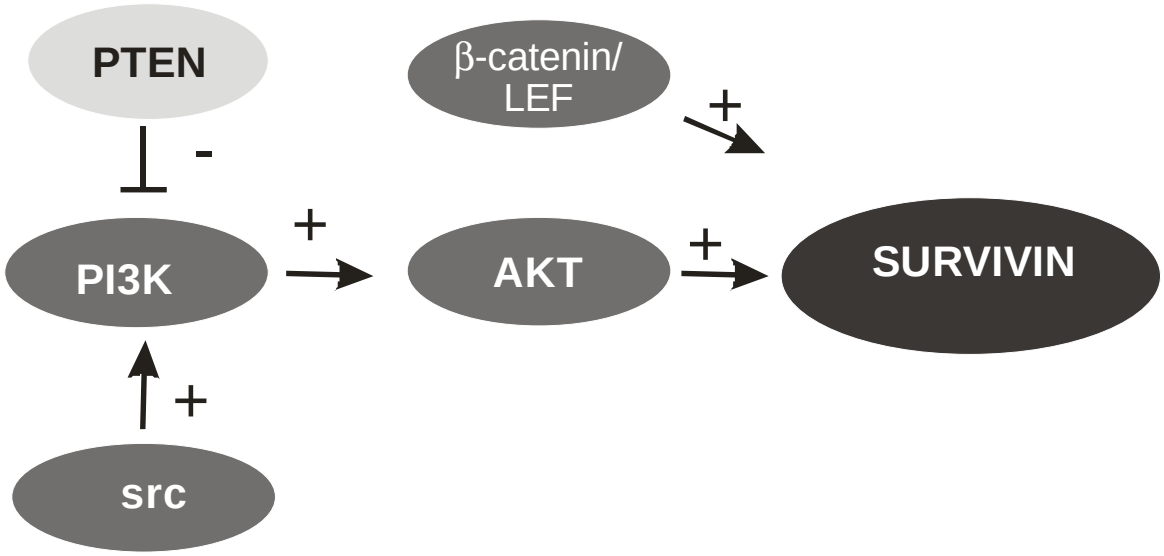


Figure 22 *The pathways regulating the expression of survivin.*

6.5 Role of E-cadherin, β-catenin and survivin on v-src transformation

E-cadherin has been shown to be a potent tumor suppressor. Its clustering at adherens junctions, together with β-catenin, leads to the binding together of neighboring cells via cell-cell junctions. This, in turn, is associated with a block of cell division and promotion of cellular polarization. Upon src transformation, this process is reversed, as was shown also in ts-src MDCK cells upon activation of src. Adherens junctions were rapidly disassembled, the E-cadherin/β-catenin complex was disintegrated, and β-catenin was released to the cytoplasm. Cytoplasmic β-catenin is translocated to the nucleus where it, conjointly with TCF, leads to the transcription of several genes, survivin among others (MacDonald et al., 2009). The high levels of survivin in v-src-transformed cells may be partly due to the free cytoplasmic β-catenin, enabling upregulation of wnt-target genes (Iurlaro et al., 2004; Torres et al., 2008; Syed et al., 2008).

6.6 Summary of the effects of 2D-3D switch and src transformation on ts-src MDCK cells in culture

Normal MDCK cells have an inbuilt polarity program to form epithelium. In 2D, they formed a monolayer of polarized cuboidal cells. In 3D, they formed cysts with a central lumen. The lumen was formed mainly by the central cells of the cyst undergoing apoptosis. The expression level of survivin, which is a major anti-apoptotic protein, was extremely low.

When ts-src MDCK cells are grown at permissive temperature in 2D, they formed a non-polarized monolayer of cells with poor cell-cell contacts reflecting deranged polarization. In 3D, however, the same cells formed cyst-like structures that were clearly different from those in normal MDCK cells. Lumen formation was deficient or lacking altogether. This depended on the type of matrix used. No lumen formation was seen in collagen I, while defective lumen formation was seen in Matrigel. Reconstitution studies showed that this difference was at least partly due to TGFβ, which was present in Matrigel but not in collagen I. The different basement membrane components seemed to be of lesser importance.

Studies with suspension cell cultures confirmed the notion that the lumen formation in 3D could be due to loss of contact with the matrix. Normal MDCK cells are sensitive to this, whereas src-transformed MDCK cells are not.

Analysis by both immunohistochemical means and gene array technology suggested that src activation affects polarization primarily by affecting the integrity of the cell-cell junctions. Survivin, on the other hand, due to its abundance in cells with complete lumen formation and complete down-regulation in src-transformed cells seemed to play a major role in the failure of apoptosis and in ‘filling of the lumen’ in the src-transformed MDCK cells.

Figure 23 summarizes the most important findings of the study concerning polarization and lumen formation in MDCK and ts-src MDCK cells in 3D.

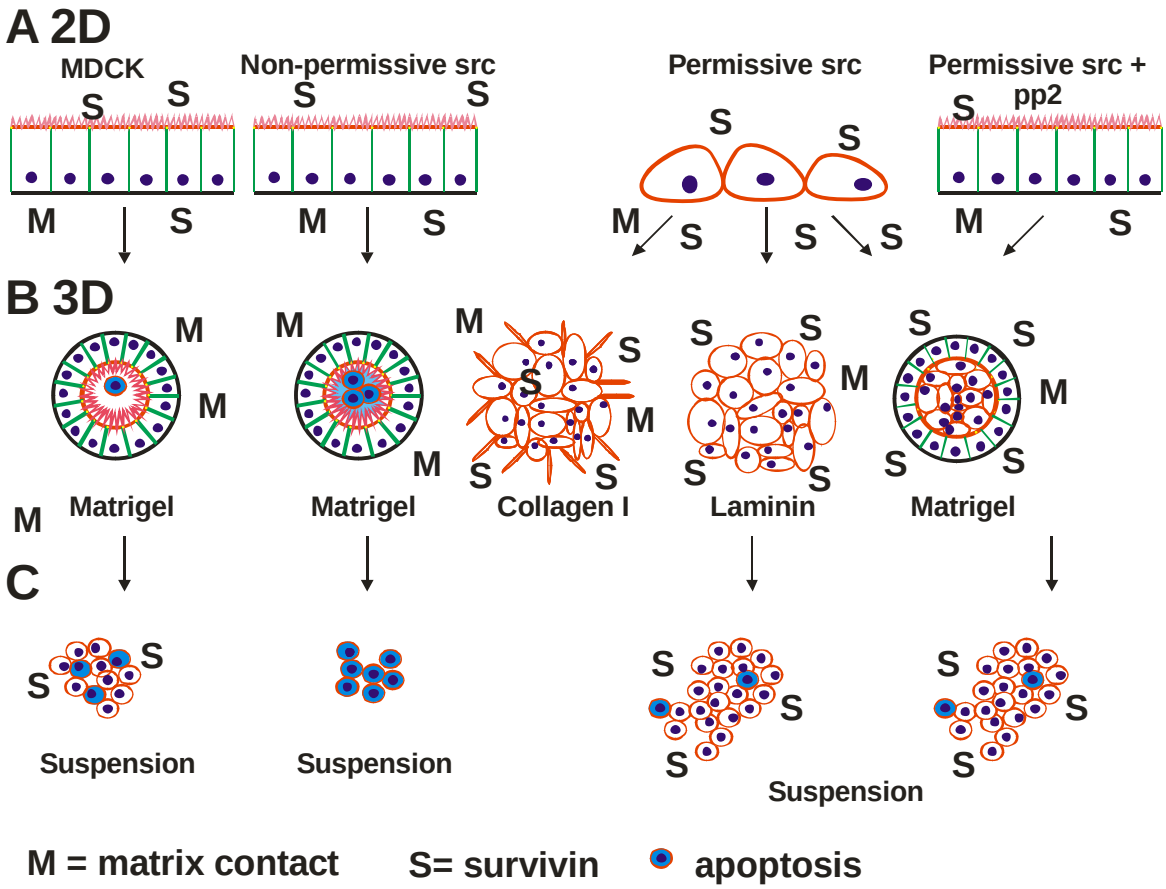


Figure 23 *The relationship between src transformation, matrix contact, expression of survivin and apoptosis. A) Cells grown in 2D conditions. All other cells except ts-src MDCK cells at permissive temperature form polarized epithelium. B) Cells grown in 3D matrices. The MDCK and ts-src MDCK at non-permissive temperature in Matrigel form a polarized cyst with a central lumen. Apoptosis is seen inside the lumina and the level of survivin is low. At permissive temperature, the morphology of the cell sphere depends on the matrix. In collagen I, the sphere has no orientation and has protrusions; when laminin is added to the culture, the protrusions vanish. In Matrigel, the cyst is filled with vital cells. The level of survivin is high and no apoptosis is seen. When pp2 is added to the ts-src MDCK cells at permissive temperature, it has no effect on polarity, the levels of survivin, or apoptosis. C) Cells grown in suspensions. Some MDCK cells go into apoptosis. All the ts-src MDCK cells at permissive temperature go into apoptosis. Most of the ts-src MDCK cells at permissive temperature avoid apoptosis. Addition of pp2 to the suspensions of ts-src MDCK cells did not induce apoptosis. The level of survivin decreases radically in ts-src MDCK cells, and all the cells are apoptotic. The change is not so radical in MDCK cells, which undergo less apoptosis. The level of survivin remains high in ts-src MDCK cells at permissive temperature. Pp2 did not affect the levels of survivin or induce more apoptosis.*

7 Summary and conclusions

Cell cultures are instrumental for studying cancer. Cell culture models are used especially to study epithelial cells, which *in vivo* form highly organized structures. In carcinomas, the polarized structure of epithelium is profoundly deranged. It is important for the cell culture models to replicate *in vitro* the actual conditions as closely as possible so as to model cancer in a controlled environment.

In this study we used ts-src MDCK cells as a model to study normal polarization and differentiation and their derangements in 2D and 3D cultures. The temperature sensitive v-src cell line is ideal for studying transformation and polarization. The transformation is transient, and can be triggered by altering the temperature of the cultures.

In the first part of the study, novel immunolabeling and imaging methods were created in order to preserve the 3D structures of the cultured cells throughout the imaging process, and to create 3D images mimicking the native structure as closely as possible. Creation of the pN1-ECad-EGFP ts-src-MDCK cell line enabled real-time monitoring of some of the key events during formation and assembly and disassembly of the adherens junctions in polarization and src-transformation in MDCK cells.

At non-permissive temperature, ts-src MDCK cells formed cyst-like structures with a central lumen that was enveloped by a layer of a fully polarized sheet of cells. At permissive temperature, this growth pattern was clearly distorted in transformed ts-src MDCK cells, but in a way that depended greatly on the composition and density or stiffness of the matrix. For example, an extreme case was a stiffer matrix made of collagen I. The transformed ts-src MDCK cells in this matrix produced cellular spheroids without a central lumen and the individual cells showed no signs of polarization. Because the individual cells displayed a motile phenotype and the lumen formation in the center of the spheroids was deficient, this kind of structure was considered to correspond to the phenomenon of ‘filling of the lumen’ seen in early stages in adenocarcinomas. The transformed MDCK cells in collagen I matrix can therefore be considered to be valid representations of adenocarcinomas *in vitro*. Another type of growth was seen in a less stiff matrix, i.e. Matrigel, in which the enveloping peripheral cell layer was polarized, but the central cavity was not fully developed, and contained viable cells with a mesenchymal or fibroblastoid phenotype. In reconstitution experiments, the essential component responsible for the counteracting effect of Matrigel on the transformation of MDCK cells, as compared with the situation in collagen I, was partly due to TGFβ1.

The studies on lumen formation in ts-src MDCK cells at non-permissive temperature demonstrated that apoptosis was the mechanism by which lumen is formed. Further studies indicated that the confinement of the apoptotic process to the center of the spheroids is due to the lack of survival-supporting matrix contact in this group of cells. At permissive temperature, in collagen I matrix, transformed MDCK cells showed no apoptosis; in Matrigel, clearly deficient apoptosis was seen, suggesting that the ‘filling of the lumen’ in this cell culture model is caused by blocking, suppressing or circumventing apoptosis.

In the second part of the study we used gene array technology to investigate how gene expression is changed in MDCK cells in response to a shift from 2D to 3D, and in

response to src transformation. Unexpectedly, when several pairwise comparisons of different growth conditions were examined, the shift in the dimensionality of culture led to at least 1.2-fold up-regulation or down-regulation of 350 genes. The genes that were most strongly affected solely by the change in dimensionality were those encoding protein involved in regulating actin cytoskeleton, vesicular transportation, cell division, and inhibition of apoptosis. This is in concordance with the changes in cell morphology and behavior, e.g. changes in the actin cytoskeleton or reduced cell division in 3D cultures, when the cells form spheres instead of a monolayer.

The genes that were most affected by the transformation via activation of src turned out to be different, depending on whether the cells were grown in 2D or 3D. In 2D, the genes that changed their expression were those encoding ECM proteins or proteins involved in immunology, e.g. MHC class II interferon, complement c 3, and those involved in the regulation of actin cytoskeleton. In 3D, the genes affected were those regulating gene expression, cell division, inhibition of apoptosis, cell metabolism, mitochondrial function, actin cytoskeleton, and encoding mechanosensitive proteins. The gene expression studies showed that activation of v-src had wide-ranging effects on gene expression, and that the genes depended crucially on the dimensionality of growth.

One of the genes most dramatically up-regulated in transformed MDCK cells in 3D was the one encoding anti-apoptotic and proliferative protein survivin. The elevated levels of survivin correlated with filling of the lumen in src-transformed MDCK cells. Further validation studies using western blotting and immunofluorescence microscopy suggested that survivin, along with its regulators PTEN and PI3K, are instrumental in counteracting the apoptotic signals which in nontransformed MDCK cells lead to lumen formation in 3D.

In conclusion:

1. In order to successfully exploit culture, immunolabeling and imaging techniques of cells in 3D, many practical problems have to be addressed. The growth conditions have to provide both correct mechanical support and distribution of ECM proteins for optimal growth and, for the immunolabeling techniques, full penetration of primary and secondary antibodies. This requires testing to find an optimal thickness and density of the gel in which the cells are grown and, preferably, conditions in which protease treatments are not needed to guarantee full exposure to antibodies and other reagents. In confocal microscopy, it is important to resort to techniques enabling rapid scanning to avoid photobleaching and the risk of misinterpreting the results.

2. 3D culture conditions are superior to 2D cultures because they provide architecturally and functionally valid representations of supracellular structures. Only in 3D matrices, do the cells form properly polarized structures and, upon transformation, mimic cancerous processes of native tissues in a relevant manner. The expression level of a vast number of genes is affected differently when the same cells are cultured in either 2D or 3D. Also the profile of the transformed genes is quite different in 2D and 3D culture conditions. Thus, the data accrued on gene expression in 2D can be informative, but are generally

quite irrelevant when compared with the findings in the more native 3D conditions.

3. The genes that were affected most by transformation of ts-src MDCK cells in 2D were the ones encoding proteins involved in immunology, actin cytoskeleton and ECM proteins. In 3D, the most affected genes were those regulating gene expression, cell division, inhibition of apoptosis, cell metabolism, mitochondrial function, actin cytoskeleton and mechanosensitive proteins. Survivin was one of the genes that underwent change in 2D, the shift from 2D to 3D, as well as src transformation in 3D. These results suggest that the effect of v-src is much more comprehensive than the enhanced or continuous activity of c-src.

4. Our gene expression analyses and biochemical and functional studies demonstrate that the inhibition of apoptosis is an independent step in the disturbed architecture of transformed MDCK cells. We were also able to show that survivin, a well-known inhibitor of apoptosis, is an important regulator in the filling of the lumen. A high level of survivin expression leads to a deficient apoptotic process which, via cavitation, leads to lumen formation in normal MDCK cells in 3D.

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